The Many Moods of Liposomes: an Investigation into Liposomes as Mediators of Difficult Immune Responses

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Abstract

Protective immunity upon vaccination is the result of complex and concerted cascades of biological events leading up to a multitude of adaptive immune responses that traditionally protect the vaccinees from infectious and toxicological danger of pathogens. Alternatively, the goal of vaccination is to generate immune responses for therapeutic treatment of established conditions, e.g. allergy and cancer. The immunity is believed to require the close cooperation of T- and B-lymphocytes and to be dependent on major histocompatibility complex (MHC)-restricted antigen presentation through antigen-presenting cells (APCs) e.g. dendritic cells and macrophages. APCs recognize antigens in tissues, process them and present the fragments in the context of MHC class I to CD8 T cells for induction of cytotoxic (CTL) responses, or in the context of MHC class II to CD4 T cells for subsequent B-cell activation. In response to peptide or protein antigens, CD4 T-helper (T_H) cells are generally required for B-cell activation and differentiation into memory cells, and for immunoglobulin class switching from IgM to IgG, IgA, and IgE. Current prophylactic vaccines primarily elicit memory B-cell responses protecting from pathogens by means of neutralizing antibodies. Allergen immunotherapy (AIT) is thought to involve antibodies, as well as T_H cells and regulatory CD4 T cells (T_{regs}), while the primary aim of cancer immunotherapy is to trigger MHC class I-restricted CTL responses as well as bystander MHC class II-restricted T_H-type 1 immune responses. Adjuvants and vaccine delivery systems enhance immune responses and have the potential to trigger specific immune responses required for protection or effective therapy. However, achieving such stimulation requires an understanding of how immunostimulators mediate their immunogenicity. Hence, the objective of this PhD thesis was to investigate the possibilities and limitations in immune-response induction with vaccines and by using liposomes, to fool the default pattern of immune-response induction and possibly pave the way for new vaccination or immunotherapeutic strategies.

Liposomal vaccine preparations were developed, produced, and tested in mice for their suitability to target antigen and adjuvant directly to B cells for stimulation of class-switched antibody responses independent on T-cell help. The T-cell independent (TI) vaccine was used as a tool to study the requirement for T cells in AIT. Alternatively, liposomes were fabricated to contain antigen and a photosensitizer for delivery of the antigen to the cytosol of APCs for stimulation of MHC class I-restricted CD8 T-cells. Finally, the delivery of immunotherapeutic antigen for AIT was achieved by routing a conventional aluminum-based allergy vaccine directly to the lymph nodes. Specifically, effects of AIT dosing intervals on the treatment efficacy were investigated.

Immunization of mice with liposomes loaded with a 15mer ovalbumin (OVA) peptide and monophosphoryl lipid A (MPLA), elicited TI IgG class switch and germinal center formation. The long-lasting antibody responses displayed characteristics of both TI-1, strictly dependent on TLR4 signaling through MyD88, and TI-2, partially

dependent on BTK signal transmission. Secondary immunization three months after the primary immunization caused a boost of all IgG subclasses. This is the first demonstration of antibody response with characteristics of both TI-1 and TI-2 type response as well as of a T-cell independent endogenous B-cell memory response *in vivo* using a peptide antigen. When the liposomal vaccine was used in a mouse model of AIT, the induced antibody responses did not protect OVA-sensitized mice from systemic anaphylaxis. However, the TI antibodies had neutralizing effects in prophylactic models of allergy and infection. When liposomes were used for targeting of the MHC class-I pathway of antigen presentation and for stimulation of CD8 T-cell responses, the combination with the photosensitizer disulfonated tetraphenyl chlorine (TPCS2a) and light treatment successfully shifted antigen processing from MHC II to MHC I, and presentation was enabled for stimulation of strong CD8 T-cell responses. Consequently, the MHC class II-dependent antibody responses were reduced in mice treated with TPCS2a-containing liposomes as compared with TPCS2a-free liposomes. Finally, when mice were sensitized to OVA and given intralymphatic immunotherapy (ILIT) with the OVA protein with 1, 2, and 4 week intervals, immunogenicity testing and tests of protection against allergic anaphylaxis suggested that longer dosing intervals improved treatment and increase both serum levels of protective IgG2a antibodies and their avidity.

In conclusion, liposomes could be successfully utilized to target B cells directly for stimulation of TI antibody responses and in a second strategy to target the MHC class-I pathway of antigen presentation for stimulation of CD8 T-cell responses. Hence, as a platform for antigen delivery, liposomes can serve very different purposes, for which reason liposomes may likely find utilization in variety of future vaccines or immunotherapies. For instance, TI vaccines may be proven beneficial for vaccination of immunosuppressed individuals, in conditions where T-cell activation has unwanted adverse effects, or during infectious outbreaks when rapid vaccination coverage and immune protection is needed. Furthermore, formulating photosensitizer in liposomes has the potential to bypass the default MHC class-II antigen presentation of vaccines antigens. The resulting stimulation of cytotoxic CD8 T-cell responses may find utilization in cancer immunotherapy or therapeutic vaccination against infections caused by intracellular pathogens that are not sensitive to and reached by antibodies. Finally, therapy intervals in ILIT affect the immunogenicity and treatment efficacy in mice and should be considered in clinical research and practice.

Zusammenfassung

Schutzimmunität bei der Impfung ist das Ergebnis von komplexen und aufeinander abgestimmten Kaskaden von biologischen Ereignissen, welche zu einer Vielzahl von adaptiven Immunantworten führen und somit die Impflinge vor einer infektiösen und toxikologischen Gefahr durch Pathogene schützt. Alternativ besteht das Ziel einer Impfung darin, Immunantworten zur therapeutischen Behandlung etablierter Zustände zu erzeugen, z.B. bei Allergien und Krebs. Es wird angenommen, dass die Immunität die enge Kooperation von T- und B-Lymphozyten erfordert und dass sie abhängig ist von der Antigenpräsentation des Haupthistokompatibilitätskomplexes (MHC) via Antigen-präsentierende Zellen (APCs), wie z.B. dendritische Zellen und Makrophagen. APCs erkennen Antigene in Geweben, prozessieren sie und präsentieren die Fragmente im Falle von MHC Klasse I den CD8 T-Zellen zur Induktion von zytotoxischen (CTL) Antworten oder im Falle von MHC Klasse II den CD4 T-Zellen für eine nachfolgende B-Zell-Aktivierung. Als Antwort auf Peptid- oder Proteinantigene werden CD4-T-Helferzellen (T_H) im Allgemeinen für die B-Zell-Aktivierung und -Differenzierung in Gedächtniszellen sowie für die Immunglobulinklassenwechsel von IgM zu IgG, IgA, und IgE benötigt. Gegenwärtige prophylaktische Impfstoffe rufen hauptsächlich Gedächtnis-B-Zell-Antworten hervor, die mittels neutralisierender Antikörper vor Krankheitserregern schützen. Es wird angenommen, dass die Allergen-Immuntherapie (AIT) sowohl Antikörper als auch T_H-Zellen und regulatorische CD4-T-Zellen (T_{regs}) involviert, während das primäre Ziel der Krebs-Immuntherapie die Induktion von MHC-Klasse-I-restringierten CTL-Antworten sowie „bystander"-MHC-Klasse II-restringierten T_H-Typ-1 Immunantworten ist. Adjuvantien und Impfstoffabgabesysteme verbessern die Immunantwort und haben das Potenzial, spezifische Immunantworten auszulösen, die für den Schutz oder eine wirksame Therapie erforderlich sind. Um eine solche Stimulation zu erreichen, muss jedoch verstanden werden, wie Immunstimulatoren ihre Immunogenität vermitteln. Das Ziel dieser Doktorarbeit war es daher, Möglichkeiten und Grenzen der Immunantwort-Induzierung mit Impfstoffen und mithilfe von Liposomen zu untersuchen, um Standard-Muster der Immunantwort-Induzierung zu täuschen und möglicherweise auch den Weg für neue Impfungen oder für immuntherapeutische Strategien zu eröffnen.

Liposomale Impfstoffpräparate wurden entwickelt, hergestellt und in Mäusen auf ihre Eignung, ob sich Antigen und Adjuvans direkt an B-Zellen zur T-Zell-unabhängigen Stimulierung von klassengewechselten Antikörperantworten richten, getestet. Der T-Zell-unabhängige (TI) Impfstoff wurde als ein Werkzeug verwendet, um den Bedarf an T-Zellen in AIT zu untersuchen. Alternativ dazu wurden Liposomen mit einem Antigen und einem Photosensibilisator, zur Abgabe des Antigens in das Cytosol von APCs, hergestellt, um die MHC-Klasse-I-restringierten CD8-T-Zellen zu stimulieren. Schließlich wurde die Verabreichung vom immuntherapeutischen Antigen für AIT erreicht, indem ein herkömmlicher, auf Aluminium-basierender Allergie-Impfstoff direkt zu den Lymphknoten geleitet wurde. Insbesondere wurde die Auswirkung der AIT-Dosierungsintervalle auf die Wirksamkeit der Behandlung untersucht.

Die Immunisierung von Mäusen mit Liposomen, die mit einem 15-mer-Ovalbumin (OVA) -Peptid und einem Monophosphoryl-Lipid A (MPLA) beladen waren, rief einen TI-IgG-Klassenwechsel und eine Keimzentrumsbildung hervor. Die langanhaltenden Antikörperantworten zeigten Eigenschaften von TI-1, streng abhängigvon der TLR4-Signalisierung durch MyD88, und von TI-2, teilweise abhängig von der BTK-Signalübertragung. Die sekundäre Immunisierung, welche drei Monate nach der Primärimmunisierung durchgeführt wurde, verursachte einen Anstieg aller IgG-Subklassen. Dies ist der erste in vivo-Nachweis einer Antikörperantwort mit Charakteristika vom TI-1- und TI-2-Typ sowie von einer T-Zell-unabhängigen endogenen B-Zell-Gedächtnisantwort unter Anwendung eines Peptidantigens. Als der liposomale Impfstoff in einem Mausmodell von AIT verwendet wurde, schützten die induzierten Antikörperreaktionen die OVA-sensibilisierten Mäuse nicht vor einer systemischen Anaphylaxie. Die TI-Antikörper zeigten jedoch neutralisierende Effekte in prophylaktischen Allergie- und Infektions-Modellen. Wenn die Liposomen zum Targeting des MHC-Klasse-I-Weges der Antigenpräsentation und zur Stimulierung von CD8-T-Zellantworten verwendet wurden, verlagerte die Kombination mit dem Photosensibilisator disulfonierter Tetraphenylchlor (TPCS2a) und eine Lichtbehandlung die Antigenverarbeitung von MHC II zu MHC I und die Präsentation ermöglichte die Stimulierung von starken CD8-T-Zellantworten. Folglich waren die MHC-Klasse-II-abhängigen Antikörperreaktionen in Mäusen, die mit TPCS2a-enthaltenden Liposomen behandelt wurden, reduziert im Vergleich zu TPCS2a-freien Liposomen. Schließlich, wenn Mäuse für OVA sensibilisiert und ihnen eine intralymphatische Immuntherapie (ILIT) mit dem OVA-Protein in 1, 2 und 4 Wochen-Intervallen gegeben wurde, zeigten Immunogenitätstests und Tests zur Evaluierung des Schutzes gegen allergische Anaphylaxie, dass längere Dosierungsintervalle die Behandlung verbessern und die Serumspiegel von schützenden IgG2a-Antikörpern und deren Avidität erhöhen.

Zusammenfassend kann gesagt werden, dass Liposome erfolgreich zur Stimulierung von TI-Antikörperantworten via das Targeting von B-Zellen verwendet werden können. Weiter kann geschlussfolgert werden, dass Liposome verwendet werden können, um den MHC-Klasse-I-Weg der Antigenpräsentation zur Stimulierung von CD8-T-Zellantworten anzusteuern. Daher können Liposome als Medium zur Antigenbeförderung sehr unterschiedliche Zwecke erfüllen, weshalb Liposome in einer Vielzahl von zukünftigen Impfstoffen oder Immuntherapien Verwendung finden könnten. Zum Beispiel können TI-Impfstoffe zur Impfung immunsupprimierter Individuen vorteilhaft sein unter Bedingungen, bei denen eine T-Zell-Aktivierung unerwünschte Effekte hat, oder während infektiösen Ausbrüchen, wenn eine schnelle Durchimpfung und Immunschutz erforderlich ist. Darüber hinaus hat die Hinzugabe von Photosensibilisatoren in Liposomen das Potential, die Standard-MHC-Klasse-II-Antigenpräsentation von Impfantigenen zu umgehen. Die resultierende Stimulation von zytotoxischen CD8-T-Zellantworten kann in der Krebsimmuntherapie oder in der therapeutischen Impfung gegen Infektionen, die durch intrazelluläre

Pathogene verursacht werden und die gegenüber Antikörpern unempfindlich sind respektive von diesen nicht erreicht werden, Verwendung finden. Abschließend beeinflussen Therapieintervalle in ILIT die Immunogenität und Behandlungseffizienz bei Mäusen, welches in klinischen Forschung und Praxis berücksichtigt werden sollte.

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Chapter 1

General Introduction

Aims and outline of the thesis

General introduction

B lymphocytes

B lymphocytes are important players in the adaptive immune system and precursors of antibody secreting cells [1]. Mature, naïve B cells are divided into distinct subsets, B-1 cells and conventional B-2 cells, the latter consisting of follicular B cells and marginal zone (MZ) B cells [2]. Follicular B cells are located in the follicles of secondary lymphoid organs such as the lymph nodes and spleen, but can circulate through the blood and lymph. They mainly participate in T-cell dependent (TD) immune responses to protein antigens [3]. MZ B cells are a mixed population of naïve B cells and memory B cells that occupy the splenic marginal zone between the marginal sinus and the red pulp, where these B cells recognize and bind to blood-borne particulate pathogens [4, 5]. In response to T-cell independent (TI) antigens, MZ B cells mediate rapid protection by differentiating into short-lived extrafollicular plasma cells [6, 7]. B-1 cells are located in pleural and peritoneal cavities and are found in small numbers in secondary lymphoid tissues. B-1 cells can be divided into B-1a, a component of innate defenses expressing CD5, and B-1b cells, which along with MZ B cells respond to TI antigens [8, 9]. B-1b cells also differentiate into extrafollicular plasma cells in a TI manner and have been shown to give rise to TI memory B cells [10] and long-lived memory plasma cells [8]. MZ B cells and B-1 cells have a lower activation threshold and rapidly differentiate into plasma cells, rendering the onset of TI antibody responses faster than TD responses, which is critical for controlling blood-borne infections [4, 11]. MZ and B-1 cells undergo positive selection based on their B-cell receptor (BCR) recognition of components of the microbial flora and self-antigens, while self-reactive follicular B cells are eradicated during their development [8].

Activation of humoral immune responses

High numbers of T and B cells accumulate and circulate in the secondary lymphoid tissues [12], a highly immunocompetent environment where antigen-presenting cells (APCs), such as macrophages, dendritic cells (DCs), and lymphocytes are brought together to initiate adaptive immune responses [13]. The close proximity of those cells allows for very efficient antigen presentation, costimulatory signaling, and bystander effects, leading to activation of T and B cells [12]. The lymph nodes have a profoundly organized structure, and a constant stream of lymph is delivered via the afferent lymphatic vessel and flows through the lymph node before draining into the efferent lymphatic vessel. This process allows for exposure to antigens, APCs, and inflammatory mediators collected in the lymph [14]. The entire lymph node is organized within a porous tissue composed of fibroblastic reticular cells, dividing it into narrow channels, providing spaces for APCs and lymphocytes to meet. Lymph nodes

have defined compartments for lymphocytes, with B cells homing to primary follicles where they survey follicular dendritic cells (FDCs) while T cells home to the paracortex and interfollicular cortex to survey DCs [15].

APCs collect antigens from tissues, internalize them and degrade to peptide fragments that can bind to major histocompatibility complex (MHC) class II molecules, and be presented on the cell surface. When APCs have sampled antigens in the peripheral tissues, they are transported in lymph to draining lymph nodes, while losing their ability to collect antigen and gaining the ability to present antigen to CD4 T cells [16, 17]. T cells make frequent contact with APCs before finding their specific antigen displayed. When the T cells encounter APCs displaying cognate antigen on MHC class II molecules, the T cells become activated and migrate to T-cell zones in secondary lymphoid organs [1, 18, 19].

B cells recognize protein antigens through their BCR while circulating in the blood. In the following, the B cells migrate to the interface of the T-cell zone and to the B-cell follicle in secondary lymphoid organs [17]. Additionally, B cells can recognize antigens presented on FDCs in primary and secondary follicles [15]. The B cells require two signals for activation of humoral responses (**Fig. 1A**). The first signal comes through the BCR and the second from activated antigen-specific CD4 T-helper (T_H) cells [20-22]. The T_H cells recognize the MHC class II-bound antigen on the B cells [22] and provide the second signal through costimulatory molecules, such as CD40, and inflammatory cytokines, such as interleukin 2 (IL-2), IL-4, IL-5, IL-6, IL-10, IL-21 and interferon (IFN) ɣ [1, 9, 19, 23, 24]. These signals promote B-cell proliferation, immunoglobulin (Ig) class switching and differentiation of B cells into short-lived extrafollicular plasma cells (PCs), early memory B cells, or germinal center (GC) B cells [9, 24-27].

B-cell activation independent on T cell help

Non-protein antigens can stimulate B cells to produce antibodies in the absence of T_H cells. Such TI antibody responses have been categorized based on their dependence on the cytoplasmic enzyme Bruton's tyrosine kinase (BTK) [28, 29]. Type 1 TI (TI-1) antigens (**Fig. 1B**) are able to elicit antibody responses in the absence of BTK, but require Toll-like receptor (TLR) signaling, whereas type 2 (TI-2) antigens (**Fig. 1C**) are fully dependent on BTK [9]. While TI-1 antigens are molecular structures specific for microbial pathogens such as lipopeptides, lipopolysaccharides (LPS), and microbial nucleic acids that provide required second signal through TLRs [30], TI-2 antigens are highly repetitive structures of polyvalent antigens such as bacterial capsular polysaccharides or viral surface proteins [31]. Through extensive crosslinking of BCRs with TI-2 antigens, BTK transmits sufficiently strong signals and thereby bypasses the need of further co-stimulatory signals [28].

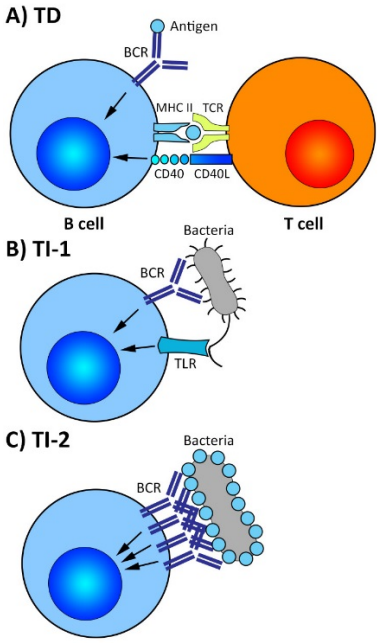

Figure 1: Classification of B-cell activation by sources of the second stimulatory signal. A) In T-cell dependent (TD) activation of B cells the second activation signal comes through CD40-CD40L ligation from CD4 T-helper cells after binding of their T-cell receptor (TCR) to antigen presented on the MHC class II molecule. **B)** In type 1 T-cell independent (TI-1) activation the second signal is provided by microbial ligands through Toll-like receptors (TLRs). **C)** In TI-2 activation polyvalent antigens transmit a strong signal through B-cell receptor crosslinking.

Germinal centers and B-cell memory

Activated, proliferating B cells form GCs in follicles of secondary lymphoid organs [32]. GCs are the sites where affinity maturation of antibodies takes place by somatic hypermutation (SHM) of the genes encoding the immunoglobulin variable region [33, 34]. Furthermore, the immunoglobulin isotype and effector function is determined by class-switch recombination (CSR) of the genes encoding the constant region [32]. Germinal centers form around six days after the first antigen encounter [35, 36]. The GC reaction peaks on days 7-10 [37], and if antigen is cleared, the GC remains for 2-3 weeks before the structure slowly involutes and disappears [25, 32, 38].

Activation of B cells by T_H cells causes the expression of the transcription repressor Bcl6 and results in proliferation and differentiation of B cells into GC centroblasts [32, 39]. Centroblasts form the dark zone of the GCs, proximal to the T-cell zone (**Fig. 2**), where the centroblasts undergo clonal expansion

and SHM. After successful affinity maturation, the centroblasts home to the GC light zone as smaller, non-dividing centrocytes. In the light zone, the centrocytes mix with a dense network of FDCs, follicular T-helper (T_{FH}) cells, and macrophages [32, 35, 40]. Expressing newly mutated antibodies, the centrocytes are able to bind antigen on FDCs for presentation to T_{FH} cells, from which they receive survival and selection signals. These signals are typically mediated through CD40L, PD-1, and BAFF, or provided by IL-4, IL-10, and IL-21 [9, 19, 23, 41], and the signals stimulate CSR and differentiation to long-lived PCs and memory B cells [42-44]. Whether the centrocytes are selected to become PCs or memory B cells is controlled by the antigen affinity. Higher affinity centrocytes differentiate to PCs, and those of lower affinity migrate to the MZ and perifollicular areas as memory B cells [45-47]. Moreover, the interaction of centrocytes and T_{FH} cells perpetuates GC reactions by stimulating centrocytes to home back to the dark zone. Here, the centrocytes again become centroblasts and can undergo further SHM. If SHM produces unfavorable antibodies and the centrocytes are unable to bind antigen on FDCs for presentation to T_{FH} cells, the centrocytes undergo apoptosis. Similarly, in the absence of T_{FH} cells, the B cells undergo apoptosis, and the GC are abrogated early after induction [25, 40]. Real-time pictures of the GC reaction have demonstrated that B cells move constantly through the GC and engage in brief but dynamic interactions with both T cells and antigens [40, 48-50].

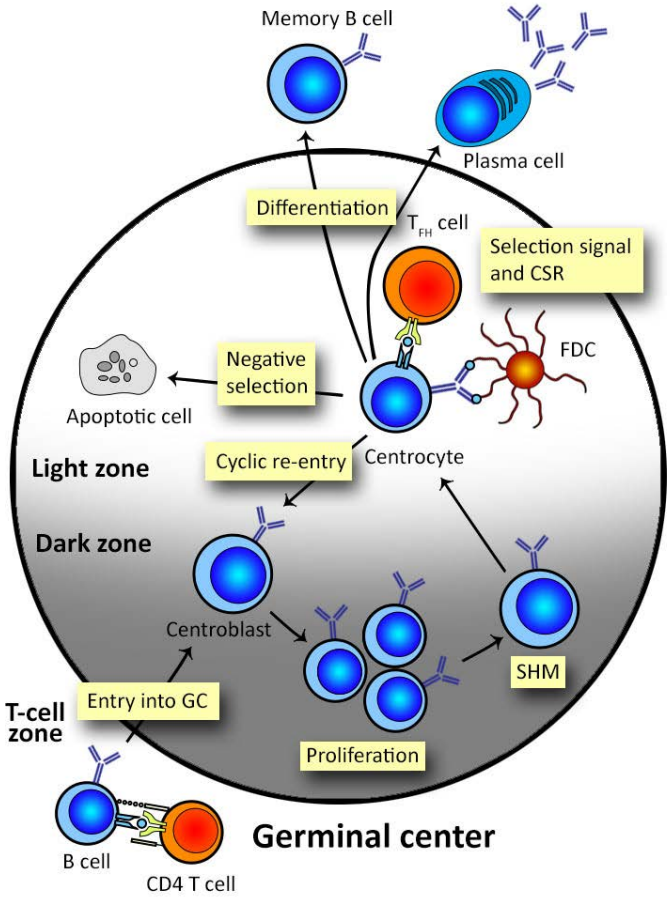

Figure 2: The germinal center (GC) microenvironment. Antigen-activated B cells differentiate into centroblasts that undergo clonal expansion (proliferation) in the dark zone of the GC. During proliferation, the process of somatic hypermutation (SHM) takes place. Centroblasts then differentiate into centrocytes and move to the light zone, where the modified antigen receptor is selected for improved binding to antigen on follicular dendritic cells (FDC) and presentation to follicular T-helper (T_{FH}) cells. Subsets of centrocytes undergo class-switch recombination (CSR) or cycle back into the dark zone for further SHM. Centrocytes producing unfacourable antibodies undergo apoptosis and are removed. Eventually, antigen-selected centrocytes differentiate into memory B cells or plasma cells.

Contrary to previous understanding, newer studies suggest that short-lived GCs can form in the absence of T cells; these studies were performed using the antigen construct of nitrophenyl conjugated to polysaccharide (NP-Ficoll) [51-53]. In part, the GC formation was obtained using transgenic quasimonoclonal (QM) mice, with high numbers of antigen-specific B-cell precursors with a uniform high affinity for NP [52, 53]. When formed in T-cell deficient mice, the GCs were small with no SHM taking place [51]. In transgenic QM mice, low mutation rates were observed in TI GCs and even in extrafollicular PCs [53]. Hypermutation was not seen in response to NP-Ficoll in wild-type mice [54], so the observed SHM might be a result of the unnatural strong signaling obtained in transgenic QM mice. GCs induced with NP-Ficoll were detected two days after immunization and were gone six days after immunization [52]. In contrast, T cells are not needed in T-cell dependent GC formation until the stage of centrocyte selection in the light zone (**Fig. 2**), approximately on day 6, after which a proportion of positively selected centrocytes cycles back into the dark zone, as centroblasts, for further SHM. The centroblasts likely undergo a finite number of divisions before they mature into centrocytes, in which case the pool of centroblasts can run out unless it is renewed [55]. The lack of centrocyte renewal in the absence of positive selection by T_{FH} cells may explain the sudden termination of the T-cell independent GC reaction, as the GC collapses by massive B-cell apoptosis a few hours after the formation of the dark and light zones [53].

Until recently, TI antigens were assumed unable to induce B-cell memory because accelerated, amplified, and affinity maturated antibody production upon secondary immunization had not been observed [8]. B-cell memory without T-cell involvement was first shown in the late seventies when memory formation was demonstrated in response to haptenated TI antigens [56, 57]. However, recall antibody responses could only be obtained when primed B cells were adoptively transferred to naïve recipients before the secondary challenge with antigen [56, 58-60]. The concept of TI memory was revisited in 2004 showing that long-lived anti-bacterial humoral immunity can be generated without T-cell help [10]. The results revealed that IgM-secreting B-1b cells from both wild type and T-cell deficient donors, that had cleared a *B. hermsii* infection, could control bacterial infection in $Rag1^{-/-}$ mice. The existence of TI memory B cells to NP-Ficoll was confirmed in the only study that has described phenotypical and functional features of TI memory B cells [61], while direct B-cell memory has only been shown in mouse models of B-cell transfer [8]. Although not proving B-cell memory, clinical vaccination with an anti-pneumococcal vaccine composed of purified TI-2 polysaccharide antigens from *S. pneumoniae* stimulated protective humoral immunity in adults [62, 63] that lasted for years [64]. Since the half-life of immunoglobulins are typically weeks, these clinical results suggest that functional memory can be obtained even with TI-2 antigens.

In contrast to B-cell memory to TD antigens, the memory induced by TI antigens is suppressed by injecting immune serum in naïve recipients before the adoptive transfer [59, 60]. TI B-cell memory has been found to be tightly regulated through negative feedback by antigen-specific antibodies [59, 61]. This negative feedback on memory B-cell responses could be a part in self-tolerance [8] or a suppressive mechanism to avoid antibody over production and to maintain humoral homeostasis [61]. If the TI memory B cells are so tightly controlled, how do they protect upon secondary infection? TI-2 antigens elicit strong and long-lasting primary extrafollicular antibody response in mice [31]. In TD responses it has been established that a second memory compartment, consisting of long-lived bone marrow (BM) PCs, is generated in addition to memory B cells [65, 66]. The BM PCs have been believed to be strictly T-cell dependent and require formation of GCs [67]. Yet, the TI-2 antigen *S. pneumoniae* capsular polysaccharide generated long-lived BM PCs secreting protective IgM and IgG [62]. Nonetheless, the PCs generated by TD and TI antigens are functionally different, suggesting that the memory PC compartment is heterogeneous [62]. It is unclear when the memory compartment comes into play upon secondary exposure to antigen, and the contribution of TI memory B cells and TI memory PCs may vary depending on antigen type and persistence as well as the route of antigen entry.

Immunoglobulins

Immunoglobulins are heterodimeric glycoproteins [68] that serve two purposes. Firstly, immunoglobulins are cell-surface receptors for antigens on B cells, permitting and transforming cell signaling that leads to B-cell activation and antibody production. Secondly, immunoglobulins are soluble antibodies, capable of binding and neutralizing antigens [69]. The immunoglobulin molecule is composed of three major fragments, two Fab fragments and the Fc fragment, consisting of two heavy chains and two light chains, with each chain containing a variable region and one or more constant region (**Fig. 3**). The paired variable regions of the two Fab fragments serve as the part of the immunoglobulin responsible for antigen recognition and confer antigen specificity. The Fc

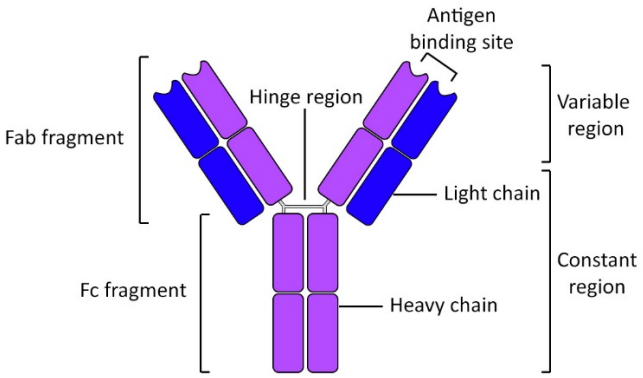

Figure 3: Structure of the immunoglobulin molecule. The immunoglobulin molecule is composed two Fab fragments and the Fc fragment, consisting of two heavy (H) chains (purple) and two light (L) chains (blue), with each chain containing a variable (V) region and one or more constant (C) regions. The two Fab fragments contain a complete L chain and the V and first C region of one H chain (C_H1). The Fc fragment is connected to the Fab fragments by a spacer hinge region between C_H1 and C_H2.

fragment is the determinant of the immunoglobulin isotype and subclass [69, 70]. There are five major immunoglobulin classes in humans and rodents, namely IgM, IgD, IgA, IgE, and IgG, the latter being the most abundant [71]. Immature B cells express IgM and IgD on their surface as they leave the BM and enter the blood and lymphatic circulations. In the absence of antigen, immature B cells survive only for a few weeks outside the BM. In response to antigen and cytokines, the immunoglobulins can undergo isotype switching to IgG, IgA or IgE. The isotypes differ in size, complement fixation and Fc receptor (FcR) binding, and different heavy chain structures results in different effector functions [69, 72].

The isotype CSR is an irreversible reaction, by which deletional DNA recombinations on chromosome 14 in humans [73] cause immunoglobulin class switch [32, 71]. Cocktails of cytokine signals or other extracellular influences variably initiate gene transcription [69, 73]. The cytokines IL-4 [74, 75], tumor growth factor (TGF)β [76, 77], and IFNɣ [78-80], as well as signals through CD40 from T cells [81] and crosslinking of surface immunoglobulins [80] regulate the CSR specificity. In addition, SHM takes place on the immunoglobulin molecules to ensure antibody diversity after exposure to antigen and by similar recombination mechanism as CSR [34]. In the case of SHM, point mutations occur in the genes encoding the V region responsible for antigen specificity as opposed to the C region in CSR. Such point mutations strengthen binding to antigen by affinity maturation of the antibody repertoire [34, 69]. B cells express the enzyme activation-induced cytidine deaminase (AID) that is required for both SHM and CSR, and only limited affinity maturation and immunoglobulin class switch takes place in the absence of AID [34, 41, 82]. Interestingly, both SHM and CSR have been shown to occur without GCs and independently of one another [31, 53]. Such distinct recruitment of AID to sites of CSR or SHM suggests that separate factors might be used by the two processes for AID recruitment, however, these factors remain unknown [34].

IgM antibodies are expressed early in B-cell development and undergo none or little SHM in response to antigen. These low-affinity antibodies are called natural antibodies and can bind invading pathogens upon entry [83]. IgD is expressed on surface of B cells as they leave the BM, and most IgD expressing cells also express IgM. Very low levels of circulating IgD is found in serum and IgD has a very short half-life [69]. Functions of both membrane-bound and circulating IgD are unclear and poorly understood, but a role of membrane-bound IgD in regulating B-cell fate at specific developmental stages through changes in activation status have been proposed [84]. CSR brings about isotype switch of the antibodies to IgG, IgA or IgE. On mucosal surfaces and in saliva and breast milk, IgA is the most abundant antibody. It provides crucial protection from toxins, viruses, and bacteria by neutralization and by preventing such agents from attaching to mucosal surfaces. In humans, there are two

subclasses of IgA, IgA1 and IgA2, differing mainly in the structure of their hinge region [69]. IgE plays a role in responses to parasitic worms and is associated with type-I hypersensitivity or allergic reactions. IgE is the least abundant isotype in serum, has a short half-life, but is a potent immunoglobulin, binding with high affinity to FcεRI on mast cells, basophils, eosinophils, and Langerhans cells. The potency of IgE is further increased when circulating IgE upregulates the expression of FcεR. In addition, IgE binds FcεRII (CD23) with low affinity [69].

IgG is the predominant immunoglobulin isotype found in the body and has the longest serum half-life. Four IgG subclasses have been identified based on structural and functional differences, IgG1, IgG2, IgG3 and IgG4 in humans and IgG1, IgG2a, IgG2b and IgG3 in mice [71]. A few inbred mouse strains, e.g. C57BL/6, also have an IgG2c gene [85, 86]. Murine immune responses usually involve all IgG subclasses and their proportion varies depending on the amount and type of antigen as well as co-stimulatory signals, which again results in distinct cytokine environments [87]. IgG antibodies can neutralize toxins and opsonize pathogens in part through activation of the complement cascade. The Fc fragment on IgG antibodies can bind and activate C1q to initiate complement-dependent cytotoxicity. Deposition of C3b further opsonizes the target, and formation of the membrane attack complex, C5-C9, causes disruption of the targeted cell membrane. Different IgG subclasses affect the downstream events of the complement cascade differently [69, 72, 88]. A key effector function for IgG antibodies is antibody-dependent cellular cytotoxicity (ADCC), in which pathogens which were recognized and bound by an antibody, bind to FcRs on natural killer (NK) cells, monocytes, macrophages, DCs, and granulocytes, which again destroy the antibody-pathogen complex through release of cytotoxic granules [69]. In addition to activating ADCC, FcR binding leads to IgG recycling, phagocytosis, cell differentiation, migration, and degranulation or secretion of biologically active molecules [89].

Mouse myeloid cells, NK cells, and B cells express ten FcRs, six of which bind IgG, and their expression is regulated by cytokines. FcRs are characterized as low-affinity receptors, binding antibodies in an immune complex, or high-affinity receptors, that also bind monomeric antibodies. High-affinity IgG-binding FcRs are FcγRI for IgG2a, FcγRIV for IgG2a and IgG2b and FcRn for all IgG subclasses [89]. Early in the immune response, when T-cell help may be limited, IFN-γ and TGF-β facilitate class switch to complement-binding IgG3 [80, 90] and IgG2b [77], respectively, that ensures early recruitment of FcγR-mediated effector functions [89]. Class switch to IgG2a occurs later, is supported by IFN-γ [91], and is associated with stronger FcγR-mediated ADCC activity than IgG2b [92-94]. Class switching to IgG1, and later to IgE is promoted by IL-4 [95, 96]. IgG1 can neutralize toxins and viruses by steric hindrance, but only engages with the inhibitory FcγRIIb to limit IgG-driven inflammatory processes. Importantly, IgG

subclasses are co-expressed and exhibit combined effector functions [87]. Human and murine FcR effector functions differ and are not fully corresponding to each other [89].

Toll-like receptors and their signaling

Toll-like receptors (TLRs) play an important role in innate and adaptive immunity by recognizing specific molecular components on microbes, so called pathogen-associated molecular patterns (PAMPs) [97]. Pathogens express various ligands that are recognized by different TLRs or other pattern recognition receptors (PRRs) such as RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs), and cytosolic dsDNA sensors (CDSs) [98-100]. TLR activation induces inflammatory responses in APCs and facilitates antigen-specific adaptive immunity [101]. TLRs are involved in different phases of the immune response, starting with pathogen recognition by APCs that causes expression of pro-inflammatory cytokines and chemokines, which again regulate DC homing to lymph nodes where antigen presentation and activation of adaptive immunity takes place [102]. Coupled with the fact that antigens alone induce weak antibody responses unless they are haptenated or aggregated [103], makes TLR ligands attractive as adjuvants in vaccines [100, 104].

The first receptor to be identified was TLR4 in 1997 [105] and to date, ten human and 12 mouse TLRs are known [98]. Most TLRs are located on the cell plasma membrane, however, TLR3, TLR7, TLR8, and TLR9 localize to endosomes [98, 106-108]. Activation of TLRs leads to recruitment of cytosolic adaptor proteins of which five have been identified: MyD88, MAL, TRIF, TRAM, and SARM [109]. These adaptors are linked to the downstream signaling pathways, which are dependent on either MyD88 or TRIF. TLR signaling leads predominantly to production of pro-inflammatory cytokines [98]. All TLRs, except TLR3 [110, 111], require MyD88, which subsequently induces NF-κB transcription and cytokine production [112]. Both TLR3 and TLR4 can utilize the TRIF signaling pathway, which can mediate activation of both NF-κB and interferon regulatory factor 3 (IRF3), leading to production of type-I IFNs [111]. Activation of TLR4 is unique in that it can use both MyD88- and TRIF to propagate immune signaling. However, it is currently unclear what dictates whether TLR4 utilizes the MyD88 or TRIF signaling pathway, and the pathways may play different roles in regulating TLR4-induced immune responses in lymphoid and myeloid cells [113]. Simultaneous activation of TLRs and the BCRs on B cells can initiate CSR of antibodies as it causes synergistic activation of canonical and non-canonical NF-κB pathways leading to AID expression. In TD responses, CD40 signaling from T cells similarly triggers NF-κB activation and AID expression, but through different signal transducers. These findings imply that TI and TD CSR require the same set of transcription factors that are critical for AID induction [114].

Vaccination

Prophylactic vaccination

Vaccination has made a major impact on global population health and successful vaccination is the most effective measure to prevent disease against future pathogens [115-118]. Not only have diseases been controlled or even eradicated, vaccines have huge socio-economic benefits and added values such as reduction in antibiotic resistance, extended life expectancy by protecting from other diseases, safer traveling, empowerment of women, decreased infant mortality, reduced health-care cost and overall an economic growth [117].

Prophylactic vaccines can be classified into two groups. The first group is comprised of live attenuated pathogens activating protective immunity similar to that seen in people who survive natural infection [119]. These vaccines protect against acute infections caused by invariant pathogens, induce potent immune responses, and have shown good efficacy with long-lived immunity. In fact, the smallpox and yellow fever vaccines confer lifelong memory and serve as gold standards [120]. The second group of vaccines includes subunit vaccines, inactivated toxins, carbohydrate vaccines and conjugate vaccines that usually contain adjuvants to enhance immune responses [119].

Current vaccines protect exclusively against diseases by eliciting antibody responses capable of reducing replication of microbes or neutralizing microbial toxins [121-123]. Efficacy of vaccination depends on the magnitude, quality and persistence of the antibodies induced. The magnitude of antibody responses is particularly important for vaccines against diphtheria, tetanus, Lyme disease, hepatitis A, polio, rabies, yellow fever, as well as *N. meningitidis*, and *S. pneumoniae* [119]. The efficacy of several other vaccines depend more on the quality of the antibody response, such as neutralizing capacity, affinity and their effector functions, which again is a result of the antibody isotype and subclass [122]. The appropriate utilization of adjuvants can help production of memory B cells and long-lived PCs, thereby shaping the antibody responses [121-123].

Despite remarkable successes in vaccine development, drawbacks such as production difficulties and safety issues, especially in immune deficient patients, has led to research and development of alternative vaccines [124]. In addition, there is a lack of effective vaccines against important human diseases. Highly variable pathogens and those causing persistent and latent infections such as HIV, hepatitis C virus and tuberculosis have been the subject of extensive research without a successful vaccine [125-127]. Development of effective vaccines has proven difficult where acute infections do not necessarily result in long-term memory and protection from reinfections, as for respiratory syncytial virus (RSV), malaria, and tuberculosis [128]. Recently, vaccines exploiting virus-like particles

(VLPs) have been marketed against human papillomavirus, Hepatitis B, and malaria, and other VLP vaccines are in clinical trials [124].

Allergen immunotherapy (AIT)

Globally, 400 million people suffer from allergic rhinitis, 300 million from asthma and nearly 250 million from food allergies, and the prevalence and impact of allergies are on the rise [129-131]. Traditionally, allergies have been associated with industrial countries, yet, reports indicate that in Asia-Pacific region and in tropical South-East Asia prevalence of allergic disease has reached the highest level in 50 years [132]. Allergen immunotherapy (AIT) is the only disease-modifying treatment with long-term effects [133]. AIT has been shown to reduce allergy symptoms and improve quality-of-life [134], as well as to reduce the risk of multiple sensitization [135], reduce rescue medication [136], and it may reduce progression to asthma [137, 138] and food allergy [139, 140].

Currently, AIT is mainly applied subcutaneously (SCIT) and sublingually (SLIT) for treatment of allergies to grass and tree pollens, pet dander, house dust mites, and venom [133, 141-143]. Both treatments consist of a dose build up, followed by 3-5 year maintenance phase. SCIT is administered by injections every 4 to 8 weeks [144], while SLIT is administered in a solution or a tablet with daily dosing regimens *[133, 136, 145]*. SCIT has "proven value", but is also associated with a 0.1-0.2% risk of severe adverse reactions [146]. SLIT was introduced as a safe and effective alternative to SCIT in the 1990ies. Oral immunotherapy (OIT) of IgE-mediated food allergy has been shown to desensitize against peanut [147], egg [148], and cow's milk allergies [149]. However, successful long-term tolerance is induced in minority of patients and adverse events are common during OIT, therefore competent personnel trained in managing anaphylaxis should administer treatment [150-152].

Important goals of current AIT research is to increase safety and to enhance efficacy [132, 133, 153]. Further emerging approaches include optimizing delivery by direct administration to immune compartments to reinforce immune responses [141, 144]. Only one in 10^7 T and B cells can recognize a specific antigen due to diverse repertoire of receptors on these cells. For this reason, antigens must be presented to millions of cells to elicit a response [12]. Since immune response requires the interaction of DCs, T cells and B cells, antigen delivery to sites where these cells are present in high numbers would facilitate reactions [12, 144]. Langerhans cells are APCs present in high numbers in the epidermis. For this reason, epicutaneous or transcutaneous AIT was introduced as a treatment option for allergies [154]. High numbers of immune cells are also present in lymph nodes for which reason intralymphatic immunotherapy (ILIT) is another attractive treatment option in allergy [12, 155]. The vaccine is injected directly into a subcutaneous lymph node under ultrasound guidance [12] and all of

the allergen is delivered to the lymph nodes [155]. Hence, the immunogenicity of a peptide-based ILIT vaccine enhances as much as million-fold compared to a SCIT vaccine [156]. The targeted delivery allows the use of lower allergen doses and fewer injections, which again should lower the incidence of local and systemic allergic side effects [144].

Allergic sensitization depends on T_H2 differentiation of allergen-specific CD4 T cells, which through release of cytokines such as IL-4 and IL-13 facilitate IgE class switching as well as development of memory T- and B-cell responses. It is the binding of allergen-specific IgE to FcεRI on mast cells and basophils which then leads to allergic sensitization [157]. Upon sensitization and memory induction, IgE-mediated elicitation of allergic reactions typically occurs in two immunological phases. The immediate phase takes place when IgE bound to FcεRI on mast cells and basophils is cross linked by allergens and is characterized by degranulation of the mast cells and basophils with release of bioactive amines, lipids mediators, chemokines and cytokines [141]. The late phase is characterized by tissue inflammation due to infiltrating T cells, and tissue injury which further trigger infiltration of granulocytes [158].

AIT can lead to long-term cease of allergy symptoms by the induction of allergen-specific immune tolerance, which can be considered as a series of changes in T- and B-cell responses as well as in the activation thresholds for mast cells and basophils [158]. Regulatory T cells (T_{REGs}) are induced early in AIT and play a pivotal role in inducing and maintaining immune tolerance by production of IL-10 and TGFβ that suppress inflammation by multiple mechanisms [159, 160]: (i) the suppression of mast cells, basophils and eosinophils [161, 162]; (ii) the suppression of APCs that support the generation of T_H1 and T_H2 cells [158]; (iii) the direct suppression of T_H1 and T_H2 cells [163]; (iv) the suppression of allergen-specific IgE; (v) the induction of IgG1 and IgG4 [164]; (vi) the indirect inhibition of T_H2-associated mucus production and activation of endothelial cells; and (vii), the T_H1-associated activation of epithelial cells and apoptosis [158].

During AIT, serum IgE increases transiently before it gradually decreases over months or years of treatment [165]. However, seasonal increases in IgE are weakened and serum IgE levels do not correlate directly with clinical improvement [166]. IgG antibodies, particularly IgG4, protect from allergic responses by binding allergen and either inhibit its binding to IgE on effector cells by direct competition, or block degranulation through the inhibitory FcγRIIB receptor [167, 168]. In addition, IgG4 antibodies can prevent binding of IgE-allergen complexes to FcεRII (CD23) on B cells, thereby reducing presentation of allergen to T cells [169, 170]. The blocking activity of IgG antibodies depends on their affinity for the allergen rather than quantity of antibodies [132, 141]. Knowledge of the

underlying mechanisms of AIT allows for development of biomarkers to predict efficacy and side effects and to monitor clinical response. Recent recommendations suggest that the efficacy of treatment correlates with the extent to which IgE can be hindered from binding to the allergen and causing an allergic response [166].

Major concerns with AIT are the incidence of adverse events as well as long duration of treatment challenging patient compliance [133, 144, 152]. Strategies aimed at improving therapy rely on modifying the allergen structure in such a way that reduces its ability to cross-link IgE, while maintaining the ability to target allergen-specific T cells and induce immune tolerance [132]. Methods changing the allergen structure include use of allergen fragments including peptides [171, 172], allergen multimers [173] and engineered recombinant hypoallergenic molecules [174], all of which would simplify standardization of allergen production and AIT safety as compared with allergen extracts [175]. Firstly, peptide immunotherapy uses short soluble synthetic peptides containing the immunodominant T-cell epitopes of major allergen proteins, but lack the length and three-dimensional structure to cross-link IgE. The use of synthetic peptides in AIT may induce antigen-specific hypo responsiveness, a shift from T_H2 to T_H1, induction of T_{REGs} and clonal deletion through exhaustion [176, 177]. Secondly, contiguous overlapping peptides fail to form the three-dimensional structure causing IgE binding and allergic reactions. Such antigens allow faster dose escalation and administration of higher allergen doses [173, 178]. Finally, recombinant allergens have great potential for increasing the safety of AIT as they can be genetically modified to reduce allergenicity and IgE binding [179]. Such an approach may ultimately allow individually tailor-made immunotherapy without the risk of novel sensitizations to irrelevant allergens present in extracts.

Immunotherapy for CD8 T-cell activation and cytotoxicity

With increased understanding of the immune system, vaccine researchers have also acknowledged the potential of cancer immunotherapy or therapeutic vaccination against intracellular pathogens [180, 181]. Such therapy aim at the induction of cytotoxic CD8 T cells (CTLs), which recognize antigens and oncogenes presented on MHC class I molecules on infected cells and tumor cells, respectively. The CTLs then kill through perforin- and granzyme-mediated mechanisms. The triggering of CTL responses requires that vaccine antigens are targeted to the cytosol of APCs, especially DCs, for correct processing in the proteasomes and access to the MHC class I pathway of antigen presentation [182]. In parallel, CD4 T helper cells are activated through MHC class II antigen presentation and provide important helper and co-stimulatory signals to the DC for efficient CTL activation [183]. The efficiency of CTL induction also depends on frequency and duration of antigen presentation making it important

for high doses of vaccine antigen to reach the cytosol of DCs. The caveat is that soluble peptide and protein antigens only poorly reach cytosol and the MHC I antigen presentation pathway [184]. Consequently, soluble vaccines induce relatively weak CD8 T-cell responses as they are rather processed through the MHC II pathway. Focus of research has therefore been directed toward the development of potent adjuvants providing cytosolic delivery and appropriate co-stimulatory signals [156].

Adjuvants

Proteins and peptides are known to have poor immunogenicity, largely due to inefficient recognition and uptake by APCs and the inability to trigger APC activation, e.g. through PRRs. Therefore, vaccine antigens are typically combined with adjuvants or delivery systems intended to enhance the magnitude and modulate the quality of the immune responses [122, 185-187]. An ideal adjuvant is safe, well tolerated, easy to scale up and manufacture, with reasonable shelf life, compatible with antigen and economically feasible [188]. Today, only few adjuvants are licensed for use in humans, namely aluminum salts (alum), adjuvant system 04 (AS04), VLPs, virosomes, and oil-in-water emulsions such as MF59 and AS03 [185, 189, 190]. Adjuvants can be particulate like alum, emulsions, liposomes, virosomes, and microparticles or combinational where particulate adjuvants have been combined with immune potentiators such as TLR ligands. AS04 is an example of such and adjuvant and is comprised of alum and the TLR4 ligand monophosphoryl lipid A (MPLA) [185]. Indeed, TLR ligands are an emerging class of adjuvants that in general stimulate APCs to prime especially T_H1 responses.

The lack of available adjuvants reflects a knowledge gap in the mechanisms of adjuvant action and potential toxic effects [122]. Currently, many types of adjuvants are being developed. One issue in adjuvant design is to control how the adjuvants stimulate specific immune responses required for protection or effective therapy, such as the stimulation of specific helper T cell subset, cytotoxic T cells or long-term memory T cells or B cells. Addressing this issue requires an understanding of how successful adjuvants mediate their immunogenicity [122].

Alum based adjuvants, are the oldest and most frequently used adjuvants in licensed vaccines [186]. Alum provides an antigen depot at the site of injection and facilitates uptake into APCs due to its particulate nature [191]. Furthermore, recent research has also shown that alum induces cell death, causing release of cellular DNA that is recognized as danger-associated molecular pattern (DAMP) by APCs [191, 192] and activation of the NLPR3 inflammasome [193]. Such an inflammatory response recruits further APCs to the site [194], thereby amplifying antigen uptake, presentation, and adaptive immune response [195]. Alum promotes a strong T_H2 response independently of TLR signaling [192,

196], and exerts a direct effect on IL-4 -producing myeloid cells essential for priming, clonal expansion, and optimal antibody production by B cells [197]. Despite the success of alum as adjuvant in prophylactic vaccines and in AIT, it has limited use in vaccines against intracellular pathogens or pathogens requiring a strong cellular immune response [186]. To overcome the limitations of alum, it has been co-delivered with other adjuvants such as MPLA in AS04, by such a combination, both T_H1 and T_H2 responses can be stimulated [198]. Alum is a commonly used adjuvant in AIT vaccines and despite its efficacy and safety, novel adjuvants are needed to overcome current problems with conventional immunotherapy. Replacement of alum with TLR ligand such as MPLA or CpG promotes T_H1 polarization and may reduce the number of injections needed in AIT [141, 144, 199].

Liposomes in antigen delivery

Liposomes are spherical particles of approximately 25-1000 nm containing one or more bilayer of phospholipids in aqueous solutions [184] and typically regarded as immunologically inert [200]. Liposomes can be effective carriers of antigens, which can be either encapsulated in the aqueous core, embedded in the lipid membrane, or absorbed on or conjugated to the liposomal surface [184]. The surface charge, rigidity, and size of the liposomes, as well as the density of antigens on the liposomal surface, influence the overall immune responses induced with antigen-loaded liposomes [184, 201]. Liposomal particles smaller than 200 nm efficiently drain through lymphatic vessels to the lymph nodes [12, 155] and thereby resemble most live attenuated vaccines [202]. While pathogens naturally display multiple PAMPs on the surface, liposomes can be engineered to carry also immune stimulatory molecules such as TLR ligands on the liposomal surface [122, 203]. Liposomes have been shown to be able to deliver antigens to the cytosol, leading to MHC I presentation and the induction of CTL responses [201]. In pre-clinical testing, multiple different liposomal vaccines have effectively stimulated antibody production sufficient to hinder infection, or induced potent antigen-specific CTL responses in cancer immunotherapy [204-206]. In addition, many vaccines have been shown to be safe and induce immune responses in clinical trials [207, 208]. Finally, a liposomal based vaccine against malaria (Mosquirix®) has been marketed [209] and virosomes, made of liposomes, are one of few licensed adjuvants and are used in hepatitis A (Epaxal®) and influenza (Inflexal®) vaccines [189].

Aims and outline of the thesis

The main objectives of the research presented in this PhD thesis were:

- ✓ to design short ovalbumin peptides for formulation into liposomal vaccines for T-cell independent stimulation of antibody responses,
- ✓ to study the mechanism by which T-cell independent liposomal vaccines stimulate antibody responses in mice,
- ✓ to investigate the role of T cells in allergen immunotherapy,
- ✓ to study the importance of dosing intervals in intralymphatic allergen immunotherapy, and
- ✓ to test the potential effect of liposomes to improve delivery of photosensitizer and antigen to the MHC I pathway for stimulation of CD8 T-cell responses.

The research in *chapter 2* focuses on the development, testing, and immunological characterization of liposomal vaccines loaded with ovalbumin (OVA) peptides and monophosphoryl lipid A (MPLA). The goal was to achieve one or several vaccines that due to the densely arranged peptides and MPLA adjuvant on the liposomal surface could activate B-cell responses directly and independent on T cells. It is generally recognized that antibody responses stimulated in the absence of T-cell help show limited isotype class switch and affinity maturation. B cells activated in the absence of T-cell help also lack a memory function in response to secondary antigen encounter. Functional B-cell memory has only been reported with polysaccharide antigens. The aim of this study was to investigate the nature of antibody responses in the absence of T cells, e.g. to elaborate on the required cellular signaling and to test the antibody function *in vivo*.

The focus of AIT research aims at improving therapeutic efficacy and safety. It is well documented that AIT induces both B- and T-cell responses, but their relative importance in AIT efficacy is frequently subject to discussions and research, for instance using murine models of T-cell depletion or adoptive transfers of B cells, T cells or antibodies in mice. The goal of *chapter 3* was to study the role of T cells in allergen immunotherapy (AIT). The above-described OVA-peptide- and MPLA-loaded liposomes were used as a tool for T-cell independent AIT, while OVA protein adsorbed on the adjuvant aluminum hydroxide (alum) was used as T-cell dependent control vaccine in AIT. As T-cell independent antibody responses arise fast and IgG class switch was feasible (*chapter 2*), it was of interest to explore the

potential of a T-cell independent allergy vaccine in AIT. The investigations were performed using an *in vivo* model of allergic anaphylaxis.

There are conflicting reports on the importance of AIT dosing intervals on the immunological and clinical effects in AIT after intralymphatic administration, so-called ILIT. One parameter that was suggested to cause conflicting results was the time interval by which the ILIT was administered. In *chapter 4*, mice were sensitized with OVA and treated by ILIT using treatment intervals ranging from one to four weeks. The therapeutic efficacy was studied using the mouse model of allergic anaphylaxis.

A limitation of current vaccines and a major challenge in vaccinology is the stimulation of T-cell responses, especially cytotoxic CD8 T-cell responses. The poor CD8 T-cell responses are in part due to non-efficient delivery of antigen to the cytosol of antigen-presenting cells (APCs) and subsequent processing in the MHC class I-restricted pathway of antigen presentation. In *chapter 5*, liposomes were utilized to facilitate delivery of an antigen and a photosensitizer to APCs *in vivo*, after intradermal administration to mice. The experimental goals were to improve APC phagocytosis and CD8 T-cell stimulation, where the photosensitizer enabled phagosomal disruption and MHC class I antigen processing upon light activation. Photosensitizer and OVA were separately formulated in liposomes, and antigen-specific T- and B-cell responses were assessed *in vivo* and *in vitro* using various immunological methods.

Finally, in *chapter 6*, the overall results and conclusions of this PhD thesis are discussed. Furthermore, the potential application of T-cell independent vaccines is discussed, and suggestions are given for further research in the field of my PhD project.

Chapter 2

A novel type of peptide-induced T-cell independent B-cell responses with class switch and memory using liposomes

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Abstract

T lymphocytes are generally required for B-cell activation and immunoglobulin class switching in response to peptide antigens. In the absence of T cells, limited IgG class switch takes place, germinal centers are short-lived, and the B cells lack memory. We show that immunization of mice with liposomes containing 15mer peptides and monophosphoryl lipid A elicits T-cell independent (TI) IgG class switch within three days as well as germinal center formation. The long-lived antibody responses were strictly dependent on MyD88 signaling and partly dependent on BTK signal transmission, thereby showing characteristics of both TI type 1 and TI type 2 antibody responses. IgG responses of all subclasses could be boosted months after primary immunization, and the biological function of the secreted antibodies was demonstrated in murine models of allergic anaphylaxis and of bacterial infection. This is the first study that demonstrates T-cell independent endogenous B-cell memory and recall responses in vivo using a peptide antigen. The stimulation of these immune responses required a correct and dense assembly and administration of peptide and adjuvant on the surface of liposomes. In the future, TI vaccines may prove beneficial in pathological conditions were T-cell immunity is compromised through disease or medicines or when rapid and antibody-mediated immune protection is needed.

Introduction

Activation of B cells and subsequent antibody production require help of CD4 T-helper (TH) cells when antigen is a protein [1]. In contrast, polysaccharides and lipopolysaccharides (LPS) can activate B cells without the help of TH cells [9]. The first step in stimulating T-cell dependent (TD) and independent (TI) antibody responses is that antigen binds B-cell receptors (BCRs). TD antigens are internalized, processed and presented to antigen-specific T cells on MHC class II molecules [210]. This process takes place in secondary lymphoid organs, where activated T cells provide stimulatory signals to B cells, which differentiate into short-lived extrafollicular plasma cells, early memory cells, or germinal center (GC) B cells [9, 35, 38, 211, 212]. In the GCs, IgM on B-cell surfaces undergo irreversible immunoglobulin class switch and affinity maturation [32], and the B cells differentiate into antibody-secreting plasma cells or long-lasting memory B cells [35, 38, 47, 213]. In general, TI antigens do not induce GC formation or memory B cells [52].

Type-1 TI (TI-1) antigens may activate B cells through signaling via Toll-like receptors (TLR) that recognize microbial ligands or synthetic ligands such as poly(I:C) [9]. In contrast, TI-2 antigens are typically repetitive, e.g. bacterial capsular polysaccharides. Such antigens extensively cross-link BCRs and deliver prolonged and persistent signals to the B cell, signals that are transmitted through the cytoplasmic enzyme Bruton's tyrosine kinase (BTK) [28, 29]. While IgG antibody responses to TD antigens are slow, TI antigens elicit fast, potent and long-lived extra-follicular antibody responses that can be critical to control blood-borne infections [4, 6, 7, 31, 51, 52].

Immunization with the TI-2 antigen NP-Ficoll have been shown to form GCs in transgenic mouse models with high numbers of high-affinity antigen-specific B cells [52, 53]. In C57BL/6 mice and in T-cell deficient mice, GCs also formed in response to NP-Ficoll [51], however, these GCs were short-lived, with limited affinity maturation and class switch [27, 51-53]. TI-associated GCs require extensive cross-linking of BCRs and are independent of CD40L or CD28 signaling [52]. In TD-associated GCs, proliferating B cells, known as centroblasts, undergo somatic hypermutation in the dark zone of the GCs. The centroblasts exit the cell cycle as smaller, non-dividing centrocytes that move to the light zone, where mixing with follicular dendritic cells (FDCs), follicular T_H (T_{FH}) cells, and macrophages [25, 32, 35, 40]. This interaction results in selection and survival of high-affinity centrocytes and provides signals for differentiation of B cells to long-lived plasma cells and memory B cells [42-44, 214].

A unique type of extra-follicular memory B cells has been identified in response to both TI-1 and TI-2 antigens [8, 10, 61]. However, recall responses to these TI antigens were only obtained in murine models of adoptive B-cell transfer [10, 56, 58-61]. The secondary activation of TI-memory B cells was

tightly regulated by antigen-specific antibodies, potentially as a self-tolerance mechanism to avoid antibody over-production [8, 59, 61]. However, protection upon secondary infection can be provided by long-lived bone marrow plasma cells independent of T cells [62]

In the present study, immunization with short peptide antigens and a TLR4 ligand densely arranged on the surface of small unilamellar liposomes activated B cells directly and independent of T cells, with generation of long-lived and class-switched antibodies, B-cell memory, and formation of GCs. The antibody responses showed characteristics of both TI-1 type reactions a TI-2 type reactions, being dependent on MyD88 and in part also on BTK signaling. To our knowledge, this is the first study that demonstrates endogenous and T-cell independent B-cell recall responses *in vivo* using peptide antigens.

Methods

Mice

BALB/c (BALB/cOlaHsd, H-2^{d}), C57BL/6 (C57BL/6JOlaHsd, H-2^{b}), and athymic nude (Hsd:AthymicNude-Fox1) mice were purchased from Envigo (Horst, the Netherlands). CBA (CBA/J, Pde6b^{rd1}), BTK-(CBA/CaHN-Btk^{xid}/J), and TRIF-deficient (C57BL/6J-Ticam1^{Lps2}/J) mice were purchased from Jackson Laboratories (Bar Harbor, Maine, USA). TLR4- (B6-TLR4^{tm1Aki}), MyD88- (B6-MyD88^{tm1Aki}), MHC class II-(B6-I-Aa^{tm1Blt}), and TCR-deficient (B6.129P2-Tcrb^{tm1Mom} Tcrd^{tm1Mom}) mice were from the Swiss immunological mouse repository (SWIMMR). Mice were kept and bred under SPF conditions at the animal facility of the *Biologisches Zentrallabor,* University Hospital Zurich. Only female mice were used, and all procedures were approved by the animal Research Ethics Board and the Zurich cantonal veterinary office (license ZH 98/2014).

Preparation of peptide liposomes

Liposomes were prepared from dimyristoyl phosphatidyl choline (DMPC), dimyristoyl phosphatidyl glycerol (DMPG), cholesterol (Solvay, Brussels, Belgium), and monophosphoryl lipid A (MPLA; Avanti Polar Lipids, Alabaster, USA) as previously described [215], and as illustrated in **Figure 1A**. Chicken ovalbumin (OVA) was used as antigen, whereby short peptides thereof were designed for formulation in the liposomes. The peptide design took into consideration immunological and physicochemical characteristics (**Table 1**), thereby avoiding known T-cell binding and IgE-binding epitopes, but including IgG-binding epitopes [216-223]. The chosen OVA-derived sequences were synthesized and flanked

with two palmitoylated lysines (Lys) on each terminal side of the peptide (PolyPeptide Laboratories, Strasbourg, France). The liposomes were loaded with 101-648 µg/ml peptide and 56-169 µg/ml MPLA.

Table 1: Peptides from ovalbumin (OVA) formulated in liposomes. Characteristics of short OVA peptides synthesized and tested for T-cell independent immunization. Exclusion criteria for the peptide selection were known T-cell or IgE-binding epitopes while peptides with known IgG-binding epitopes were included [216-223]. Minor amino acid mutations were created to assure peptide stability and solubility. The peptides were synthesized and flanked with two palmitoylated amino acids (Lys) on both the N- and C-terminal side. The fatty acid-derived palmitoyl group facilitated anchoring of the peptide in the bilayer of liposomes [224]. The N-terminal amino group of all the peptides was kept free while the C-terminal carboxylic group was capped as amide.

Code	Amino acids	Sequence OVA aa	Length (aa)	Aliphatic Index[1]	Instability Index[2]
OVA1	H-Lys(Pal)-Lys(Pal)-**FKELKVHHANENIFY**-Lys(Pal)-Lys(Pal)-NH2	15-29	15+4	61.58	32.91
OVA2	H- Lys (Pal)- Lys (Pal)-**RFDKLPGFGDSIEAQ**-Lys(Pal)-Lys(Pal)-NH2	58-72	15+4	46.32	67.59
OVA3	H-Lys(Pal)-Lys(Pal)-**TKPNDVYSFSLASRY**[3]-Lys(Pal)-Lys(Pal)-NH2	91-105	15+4	41.05	7.71
OVA4	H-Lys(Pal)-Lys(Pal)-**RNVLQPSSVDSQTA**-Lys(Pal)-Lys(Pal)-NH2	158-171	14+4	59.44	97.67
OVA5	H-Lys(Pal)-Lys(Pal)-**RNVLQPSSVDSQTAMVLV**-Lys(Pal)-Lys(Pal)-NH2	158-175	18+4	92.73	80.42
OVA6	H-Lys(Pal)-Lys(Pal)-**VFKGLWEKAFKDEDTQA**-Lys(Pal)-Lys(Pal)-NH2	179-195	17+4	41.9	-3.18

[1] The aliphatic index is regarded as a positive factor for solubility of proteins [225].

[2] The instability index has been shown to inversely correlate with *in vivo* half-life [225].

[3] Original sequence was **TKPNDVYSFSLASRLY**

Immunization of mice

Typically, groups of five mice were immunized subcutaneously (s.c.) in the scruff of the neck with OVA-derived peptide- and MPLA-loaded liposomes or OVA on alum. A single dose ranged from 2-36 µg peptide and 1.3-25.6 µg MPLA, and the liposomes were diluted in phosphate-buffer saline (PBS) prior to injection. T-cell dependent control vaccines were made using full Grade V OVA protein from Sigma-Aldrich (Buchs, Switzerland) mixed with aluminum hydroxide (Alum; Alhydrogel 2%) from Brenntag Biosector (Frederikssund, Denmark). OVA was allowed to absorb on alum for one hour at room temperature, before dilution in PBS. The OVA doses ranged from 10 to 100 µg OVA and the alum dose was kept at 1 mg. When indicated, the immunization was repeated, and mice were bled from the tail vein for later antibody analysis by ELISA.

Measurement of OVA-specific antibody responses by ELISA

OVA-specific IgM, IgG1, IgG2a, IgG2b, IgG3, and IgE antibody responses were determined by a sandwich ELISA [226]. Briefly, the plates were coated with 50 µl 4 µg/ml OVA for IgM and IgG subclass detection and with 3 µg/ml anti-mouse IgE (AbD Serotec, Düsseldorf, Germany) for detection of IgE. OVA-specific IgE was detected with an in-house biotin-conjugated OVA, while IgM and IgG subclasses were detected with biotin-conjugated goat anti-mouse IgM or IgG subclass-specific antibodies (Abcam, Cambridge, United Kingdom), respectively. All plates were developed with streptavidin-conjugated horseradish peroxidase (HRP) and with TMB substrate (eBioscience, San Diego, CA, now Thermo Fischer). Reaction was stopped with 25 µL of 2N sulphuric acid (H_2SO_4) and absorbance read at 450 nm on a BioTek Instruments plate reader (Winooski, VT). Antibody titers were defined as the highest dilution with absorbance two times that of sera from untreated mice, or optical density (OD) of 0.1. Alternatively, the OD at a given, non-saturated serum dilution was measured for IgG2b, IgG3 and IgM, while the serum concentration of OVA-specific antibodies was calculated against standards for IgG1, IgG2a (BioLegend, San Diego, CA) and IgE (AbD Serotec).

ELISA for avidity measurements

The avidity of serum antibodies were measured in three steps as previously described [227-229]. Firstly, the ELISA was optimized so that the amount of serum antibody binding the OVA-coated plate represented a small portion of the amount of antibody in the non-binding liquid phase. The amount was equivalent to a developed ELISA of OD 0.2. Secondly, each individual test serum was diluted to obtain the optimal antibody concentration (OD 0.2). Thirdly, the diluted sera were incubated at room temperature with OVA protein at a range of OVA concentrations and for two hours. The sera were then transferred to OVA-coated plates and the ELISA developed. The concentration of OVA needed to inhibit 50% of the antibodies from binding to the plate (IC_{50}) was calculated by "log (inhibitor) vs response" fitting using GraphPad Prism 7.02 (GraphPad Software Inc., La Jolla, CA). Sera from individual mice within each treatment group was pooled to yield one sample per group.

Germinal center analysis by immunohistochemistry, immunofluorescence and flow cytometry

BALB/c mice were immunized intravenously (i.v.) with 10 µg peptide-loaded liposomes. As a positive control for germinal center (GC) formation, 10 µg Qβ virus-like particles (VLPs) was administered [230]. Spleens were harvested for the analysis of GCs 5, 8, 13 and 20 days after injection, and the tissues were snap-frozen for immunohistochemistry (IHC). The frozen sections were stained with peanut agglutinin (PNA) and anti-B220. Athymic nude mice were immunized in the same way, and spleens were harvested 3, 5, and 10 days after injection and snap-frozen for staining by immunofluorescence (IF).

The frozen sections were stained with PNA-Dylight549, anti-B220-Dylight488 and DAPI. All stainings were outsourced to Sophistolab AG (Muttenz, Switzerland).

Alternatively, spleens were harvested seven days after injection with peptide-loaded liposomes or with VLPs and analyzed by flow cytometry after RBC lysis, FcR blocking with anti-CD16/32 antibodies, and staining with fluorescently labelled anti-B220, anti-GL-7, and anti-CD38 (all eBioscience). Germinal center B cells were defined as positive for B220 and GL-7 and negative for CD38. Acquisition was done with FACSCanto (BD Bioscience, Basel, Switzerland) and analysis with FlowJo 10.0.8 (FlowJo, LLC, Ashland, OR).

Analysis of systemic anaphylaxis

BALB/c mice were immunized s.c. with 10 µg peptide-loaded liposomes or with 100 µg OVA protein on alum, as described above. On week 4, a booster vaccine was given. On weeks 8 and 10, all mice received s.c. injections of 100 µg OVA on alum in 100 µL PBS as to produce OVA-specific IgE sensitization. On week 14, mice were challenged by intraperitoneal (i.p.) injection of 50 µg OVA in 100 µl PBS, and anaphylaxis was analyzed by measuring the rectal temperature before and after the challenge using a digital thermometer (Thermalert TH-5 with a RET-3 probe, Physitemp, Huron, NJ).

Adoptive IgE sensitization of nude mice by serum transfer

As IgE class switch is dependent on T cells [231], nude mice cannot be IgE sensitized. Therefore, athymic nude mice were adoptively transferred with serum from IgE-sensitized BALB/c mice. Twenty hours after serum transfer, the nude mice were challenged for induction of anaphylaxis as described above. One group of nude mice was immunized s.c. with 10 µg peptide-loaded liposomes 3 and 7 weeks prior to serum transfer. The goal of this experiment was to test the functionality of TI antibodies.

Infection of mice with Listeria monocytogenes

To investigate if immunization with the peptide- and MPLA-loaded liposomes could control acute bacterial infections, BALB/c mice were immunized s.c. with 10 µg of peptide- and MPLA-loaded liposomes as described above. Booster immunization were given when indicated, and blood was collected for detection of antigen-specific antibodies. One to three weeks after the last immunization, the mice were infected with OVA-expressing and streptomycin-resistant *Listeria monocytogenes* [232], kindly provided by Dietmar Zehn (School of Life Sciences Weihenstephan, Technical University of Munich, Germany). Briefly, bacterial stocks were grown to mid log phase in brain-heart infusion (BHI) broth, and the bacteria quantified by measuring the OD at 600 nm. Mice received 10.000 colony-forming units (CFU) in PBS by i.v. injection, and the bacterial burden in spleen and one liver lobe was

analyzed after 48 hours. Organs were collected in PBS, mashed through a 70 µm cell strainer and homogenates incubated with 2.5% saponin (Sigma-Aldrich) for 60 minutes at room temperature before serial dilutions of organ lysates were spread onto BHI agarose plates supplemented with 200 µg/mL streptomycin. After 48 hours of incubation at 37°C, the CFU were counted and the organ bacterial burden calculated.

Statistical analysis

The statistical analysis were performed using GraphPad Prism 7.02 software. The non-parametric data were analyzed by Kruskal-Wallis analysis for multiple comparisons or by Mann-Whitney analysis for analysis of two groups. The significance level was set at 0.05.

Results

Ovalbumin peptide-loaded liposomes induced OVA-specific antibodies and IgG class switch

Immunization of BALB/c mice with the peptide- and MPLA-loaded liposomes (**Fig. 1A**) triggered OVA-specific IgG isotype switch with production of IgG1, IgG2a, IgG2b, and IgG3 subclasses (**Fig. 1B**); the antibodies were specific also for the individual OVA-derived peptides loaded on the liposomes (data not shown). Among the peptide-loaded liposomes, those with the 15mer aa58-72 (OVA2) and the 17mer aa179-195 (OVA6) were the most immunogenic vaccines. The level of antigen-specific IgG2a and IgG2b antibodies secreted was typically comparable to the levels obtained using a TD vaccine based on OVA protein and alum. OVA on alum was significantly more immunogenic for IgG1 production ($p=0.0022$), while the OVA2-liposomes induced significantly stronger IgG3 responses ($p=0.0043$). While the protein vaccine stimulated OVA-specific IgE, the liposomal peptide vaccines did not (**Fig. 1B**). Of note, when BALB/c mice were immunized with a single injection of 4 µg soluble OVA2 or OVA6 peptides admixed to MPLA-loaded liposomes, no antibody responses could be detected (data not shown). Subsequent experiments were done using the OVA2 formulation.

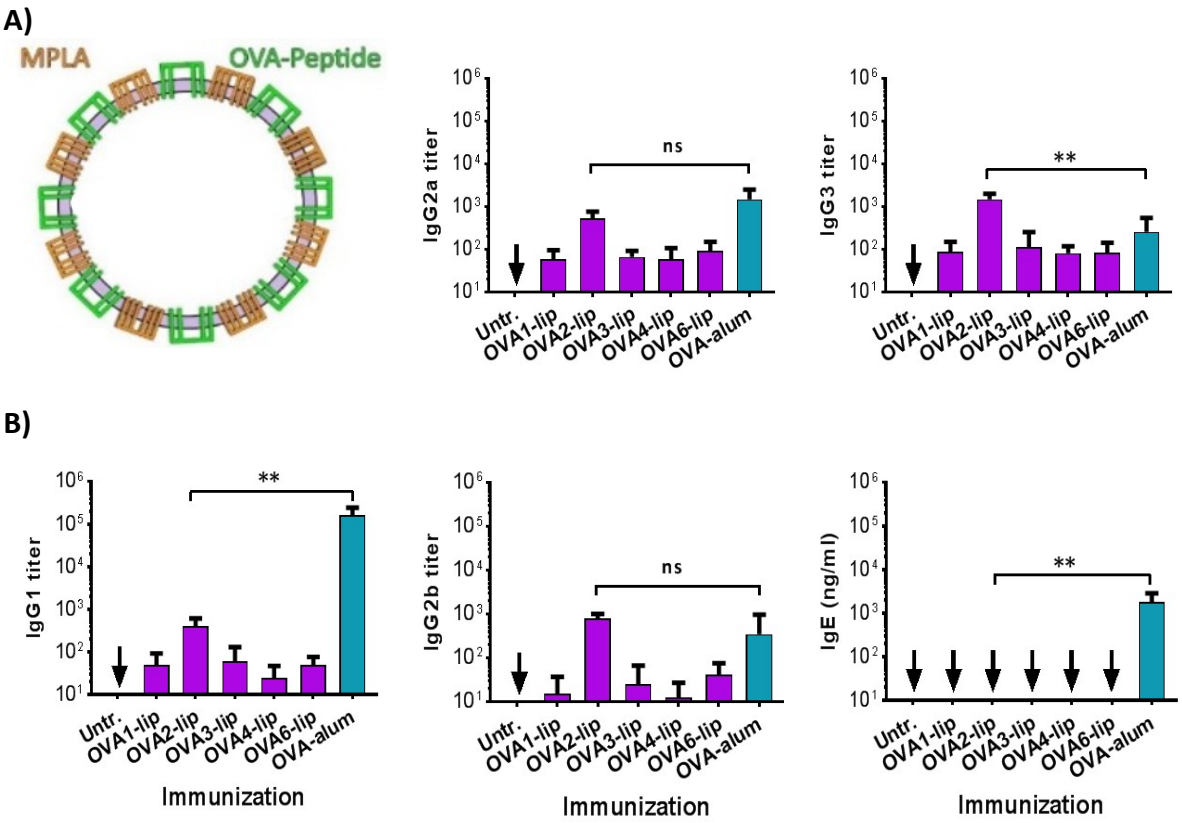

Figure 1: Peptide- and MPLA-loaded liposomes stimulate IgG class switch in vivo. (**A**) Schematic representation of ovalbumin (OVA) peptide- and MPLA-loaded liposomes. The OVA-derived peptide is anchored in the liposomal membrane by palmitoyl chains coupled to the side chains of two lysines on both ends of the peptide. The adjuvant monophosphoryl lipid A (MPLA) is also integrated in the liposomal membrane through its six acyl chains. (**B**) OVA-specific IgG1, IgG2a, IgG2b, IgG3, and IgE antibodies in sera from BALB/c mice immunized on days 0 and 7 with the different peptide-loaded liposomes or OVA protein absorbed on alum. Sera was collected on day 14. Arrows indicate sera not reaching the detection limit of OD 0.1 for a serum dilution of 1/50. Results are expressed as mean + SD (n = 6 per group) and the data are representative of four independent experiments with comparable results. ** $p < 0.01$ calculated by two-tailed non-parametric Mann-Whitney U test, comparing OVA2-lip and OVA-alum.

Peptide- and MPLA-loaded liposomes stimulated antibody responses independent on T cells

T-cell deficient athymic nude mice immunized with the OVA2-liposomes produced strong OVA-specific antibody responses with immunoglobulin class switch to all IgG subclasses (**Fig. 2A**). The antibody responses were comparable to those determined in immune competent BALB/c mice. To confirm T-cell independency, MHC class II-deficient mice lacking mature CD4 T cells and TCR-deficient mice lacking both αβ and γδ T cells were immunized with liposomes, and the induced OVA-specific IgG responses were comparable to antibody responses in wild-type C57BL/6 mice (**Fig. 2B**). The robust antibody responses in the T-cell deficient mouse models and the strong IgG2b and IgG3 production

demonstrate that both antibody responses and IgG class switch can be obtained in the absence of T cells.

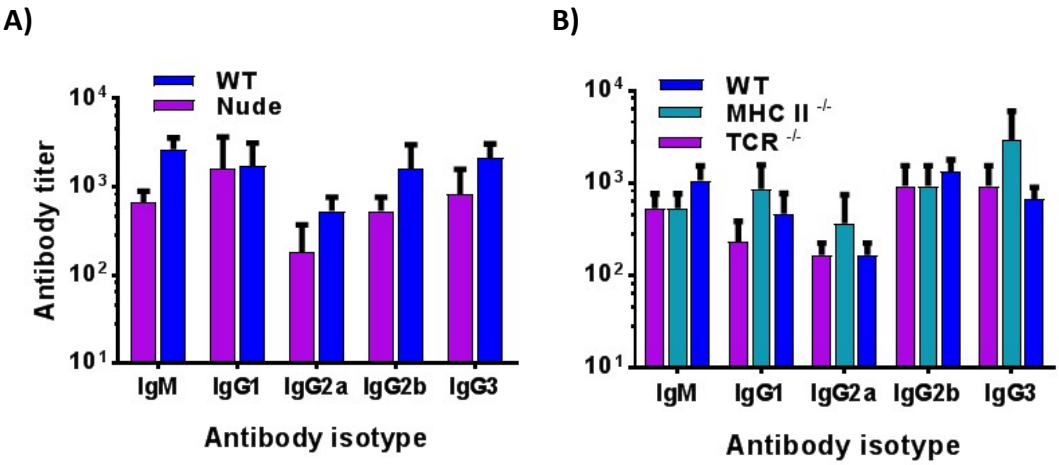

Figure 2: Antibody levels are comparable in wild type and T-cell deficient mice. (**A**) OVA-specific antibody titers measured in sera from athymic nude and BALB/c wild type (WT) mice 6 weeks after immunization with OVA2 peptide- and MPLA-loaded liposomes in weeks 0, 1, 2 and 3. (**B**) Antibody titers in TCR-deficient, MHC class II-deficient, and C57BL/6 WT mice measured in sera 7 weeks after immunization with OVA2-loaded liposomes in weeks 0 and 1. C57BL/6 mice were immunized in weeks 0, 1, 2 and 3 and serum collected three weeks after last immunization. Results are expressed as mean + SD (n = 3 per group).

Immunization with peptide- and MPLA-loaded liposomes triggered fast antigen-specific antibody production as well as formation of germinal centers independent on T cells

The OVA2-liposomes stimulated detectable IgM production within two days of immunization in BALB/c mice (**Fig. 3A**). The IgM response peaked on days 4-6, after which it declined. The IgG1 and IgG3 class switch started on day 3, and serum IgG3 kept rising through day 9. The IgG2a and IgG2b serum conversion were the slowest with a switch on days 4 to 6, respectively, but both subclasses kept rising through day 9.

Immunization of BALB/c mice with liposomes also caused formation of GCs in the spleens as determined by histological PNA (**Fig. 3B**) and B220 (**Fig. 3C**) staining in the spleen follicles. The GCs were detected five days after immunization and they remained as long as 15 days (not shown). Formation of GCs was also observed in athymic nude mice as early as three days after immunization with liposomes (**Fig. 3D**), and these TI-associated GCs remained for at least 10 days (**Fig. 3E**). The GCs induced by peptide-loaded liposomes, in both BALB/c and nude mice, were of comparable size to GCs induced by immunization with Qβ VLPs, which were used as a positive control for GC formation. However, the number of GCs were generally lower after immunization with liposomes than after VLPs.

Flow cytometry analysis of spleens from BALB/c mice confirmed GC formation after immunization and revealed increased expression of GL-7-positive CD38-negative B cells compared to naïve mice (**Fig. 3F**).

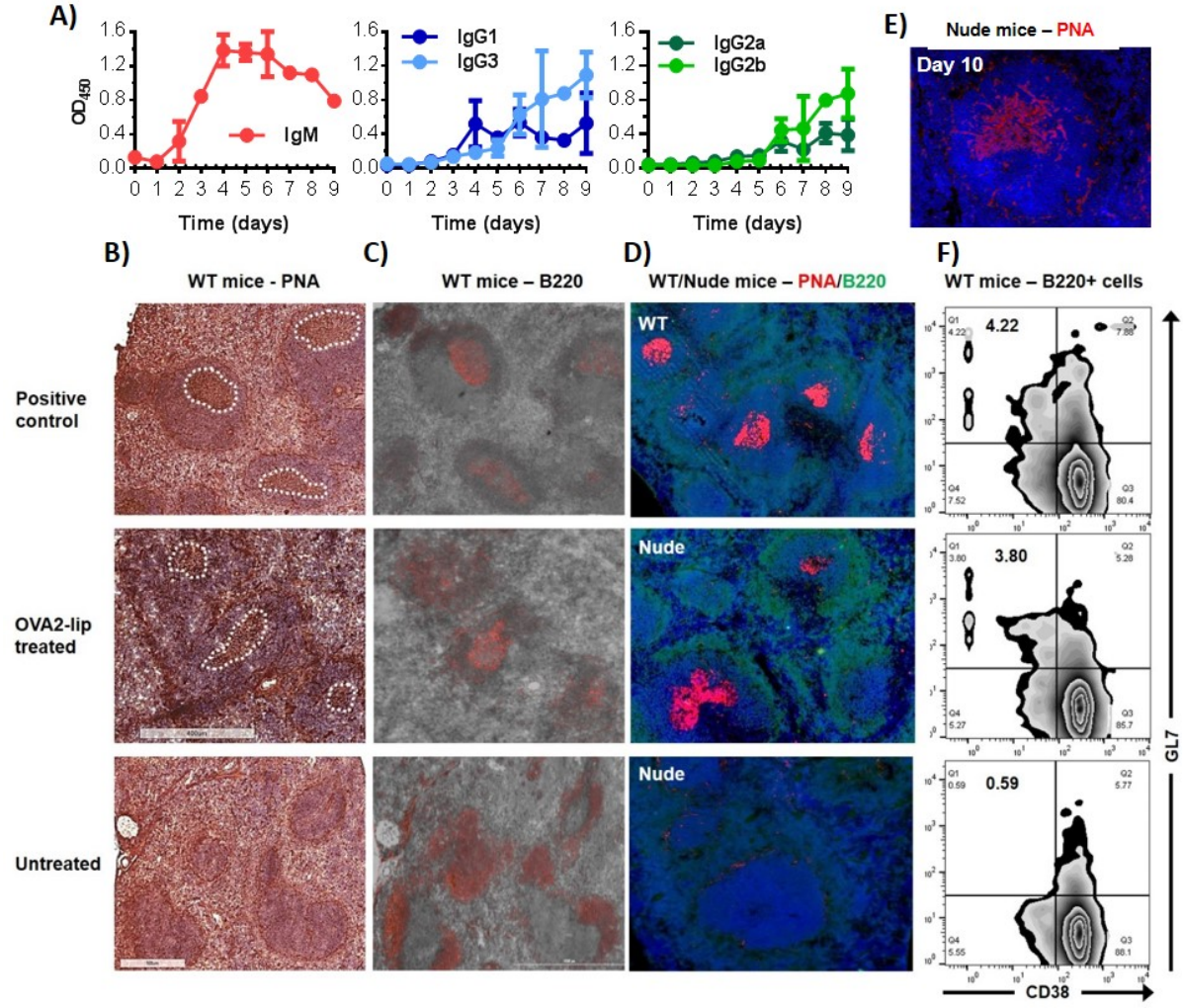

Figure 3: Rapid antibody production and long-lived germinal centers independent on T cells. (**A**) OVA-specific antibody levels in BALB/c mice after an intravenous injection of 10 µg OVA2 peptide- and MPLA-loaded liposomes. Serum was diluted 1/125 and levels are expressed as mean OD ± SD (n = 2 per group). (**B-F**) Formation of germinal centers after immunization with 10 µg OVA2 liposomes or 10 µg Qβ VLPs as a positive control in BALB/c (**B, C, F**) and nude (**D, E**) mice. Spleens from BALB/c mice were analyzed by immunohistochemical staining with PNA (**B**) and B220 (**C**). The OVA2-lip treated samples is from day 13 and the positive control from day 5. (**D, E**) Spleens from nude mice were analyzed for germinal center formation by immunofluorescence staining with PNA (red), B220 (green) and DAPI (blue). The OVA2-lip treated samples are from days 3 (**D**) and 10 (**E**), and the positive control from a BALB/c mouse on day 10. (**F**) Representative zebra plots of GL-7 and CD38 expression on B220 positive BALB/c lymphocytes from day 7. Germinal center cells are defined as GL-7 positive and CD38 negative.

Immunization with peptide- and MPLA-loaded liposomes triggered formation of B-cell memory and affinity maturation

After a single injection of the peptide-loaded liposomes, high levels of antibodies of all IgG subclasses were detected in serum for at least 14 weeks, and the antibody longevity was not dependent on T cells (**Fig. 4A**). A strong and T-cell independent IgG boost was observed upon secondary immunization. Indeed, the IgG1 antibody responses to peptide-loaded liposomes were higher in nude mice than in BALB/c mice, while results from all other subclasses revealed that neither antibody levels nor the memory or recall properties were affected by T cells (**Fig. 4A**). As expected, OVA on alum triggered higher levels of IgG1 and IgG2a (**Fig. 4B**) antibodies than did peptide-loaded liposomes. While IgG2b antibody responses were comparable, limited IgG3 boosting was observed after immunization with OVA on alum.

The avidity of antibodies from immunized BALB/c and nude mice was evaluated by competitive ELISA as a measure of affinity maturation. The tested sera were collected 1 and 14 weeks after primary immunization, and 8-9 weeks after a secondary immunization (weeks 22-23). The avidity of the antibodies was measured as the concentration of OVA needed to inhibit 50% of the antibodies from binding to plate-coated OVA. Antibodies of higher avidity bind more strongly to OVA, hence, lower OVA concentrations are needed for inhibition. The antibody avidity increased from week 1 to week 14 for all antibody subclasses and all treatment groups (**Fig. 4C**). A second immunization did not increase antibody avidity, despite the strong antibody boosting observed (**Fig. 4A-B**). The avidity measured for antibodies after immunization with OVA on alum was higher than with peptide-loaded liposomes, even for the IgG2b and IgG3 (**Fig. 4C**). The antibody levels (**Fig. 4A**) as well as avidity (**Fig. 4C**) were comparable in wild type BALB/c and in athymic nude mice following liposomal immunization.

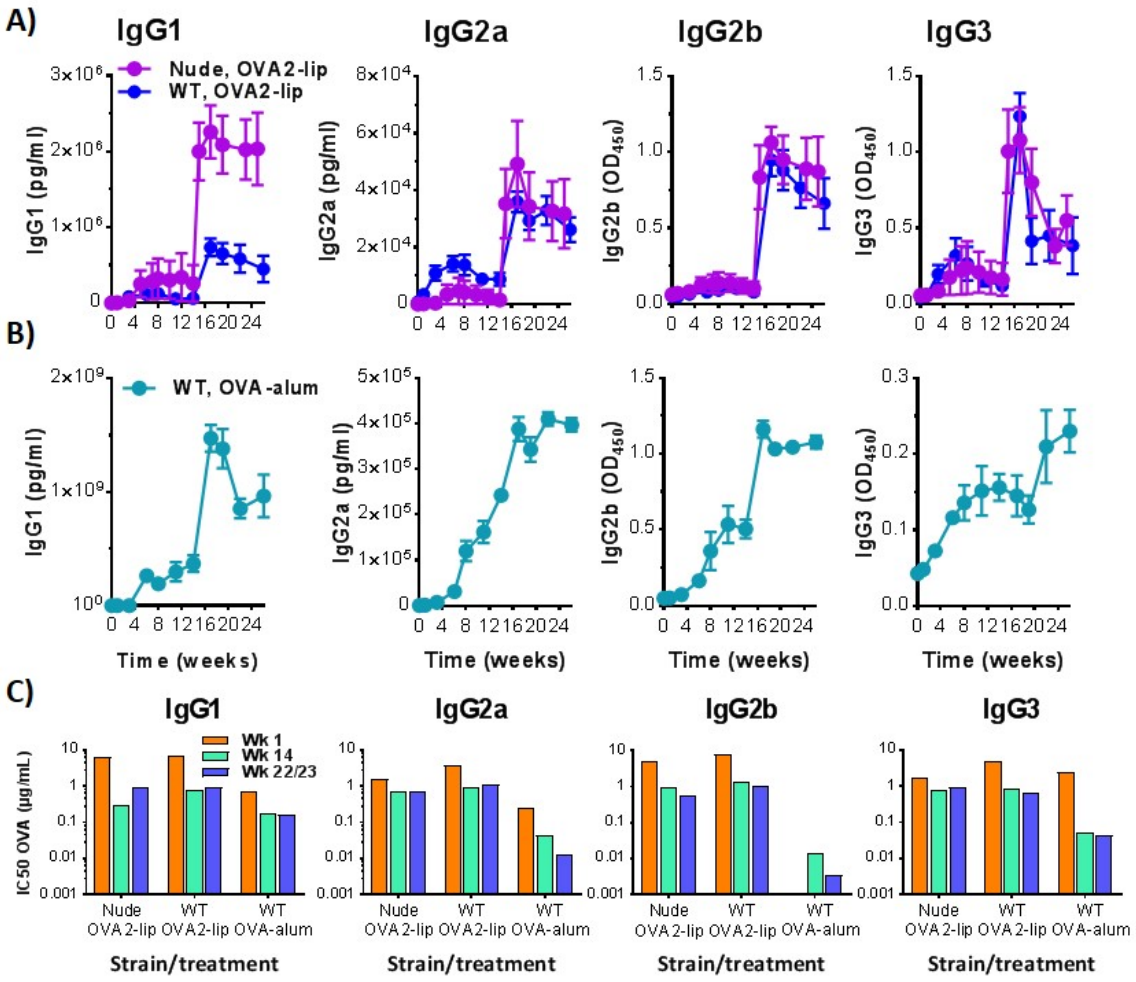

Figure 4: Antibodies are long-lived with affinity maturation and B-cell memory responses taking place independent on T cells. Production of OVA-specific IgG1, IgG2a, IgG2b and IgG3 antibodies in wild type (WT) BALB/C and in nude mice after immunization with (**A**) 10 µg OVA2 peptide- and MPLA-loaded liposomes or (**B**) 100 µg OVA on alum on weeks 0 and 14. Results are expressed as mean ± SEM (n = 5 per group). (**C**) Avidity of IgG1, IgG2a, IgG2b and IgG3 antibodies in sera collected in weeks 1, 14 and 22 (WT) or 23 (nude) analyzed by competitive ELISA.

Antibody responses induced by peptide- and MPLA-loaded liposomes displayed characteristics of type 1 and 2 T cell independent responses

We further investigated the nature of the second signal for T-cell independent B-cell activation. The adjuvant MPLA can bind TLR4 and, in fact, antibody production was completely abrogated in TLR4 deficient mice (**Fig. 5A**). The TLR4 signals were transmitted through the adapter molecule MyD88 but not through TRIF, as antibody production was abrogated in MyD88- but not in TRIF-deficient mice (**Fig. 5B**).

In type 2 TI (TI-2) antibody responses, a co-stimulatory signal for B-cell activation comes through the cytoplasmic enzyme BTK following extensive cross-linking of the BCRs. We hypothesized that the high density of peptides on the liposomal surface could cross-link the BCRs. Indeed, antibody production and class-switch was impaired in BTK-deficient mice immunized with the peptide-loaded liposomes and only IgM and IgG2a antibodies were detected (**Fig. 5C**).

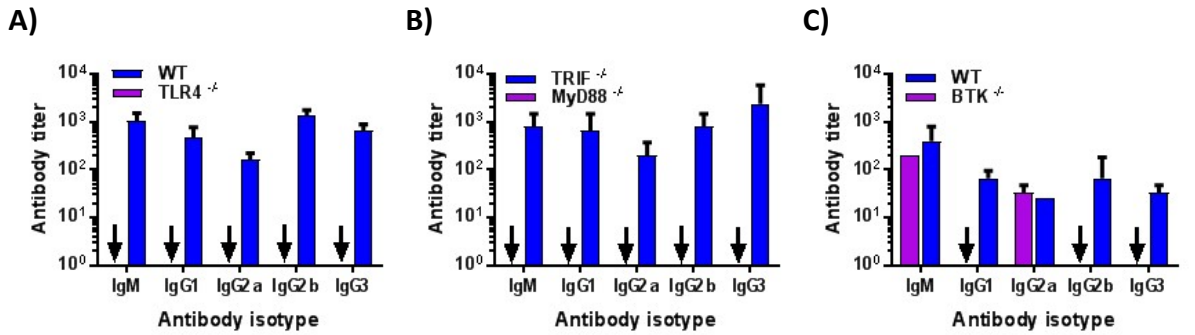

Figure 5: Antibody responses are dependent on TLR4 signaling through MyD88 and partially on BTK. (**A**) OVA-specific antibody titers in (**A**) wild type (WT) C57BL/6 and TLR4-deficient mice or in (**B**) MyD88 and TRIF deficient mice 6 weeks after two weekly immunizations with the OVA2 peptide- and MPLA-loaded liposomes. The WT mice were immunized in weeks 0, 1, 2 and 3 and serum collected in week 6. (**C**) Antibody titers in WT CBA mice and in syngeneic BTK-deficient mice 6 weeks after two weekly immunizations with the OVA2 liposomes. Arrows indicate sera not reaching the detection limit. Results are expressed as mean + SD (n = 3 per group).

Antibodies induced with peptide- and MPLA-loaded liposomes are functional and protect mice against allergic anaphylaxis

To test the biological function of antibodies induced by peptide-loaded liposomes, mice were immunized, then IgE-sensitized to OVA protein, and finally challenged with a high dose of non-adjuvanted OVA intraperitoneally (**Fig. 6A**). Sensitized mice react with an anaphylactic shock manifesting as a drop in body temperature. When mice were immunized with peptide-loaded liposomes 14 and 10 weeks before the challenge, they reacted with less anaphylactic symptoms and less severe temperature drops than did non-immunized mice (**Fig. 6B**). Indeed, the protection was comparable to that obtained in mice immunized with OVA on alum. The maximum average temperature drops were 6.2 °C for non-immunized mice, 3.1 °C (OVA on alum) and 3.6 °C (OVA2 liposomes) for immunized mice. When calculating the integral area above curve (AAC) for the change in body temperature and as defined by time-temperature function and the baseline temperature, immunization with OVA on alum or peptide-loaded liposomes produced comparable protection (**Fig. 6B, right panel**). However, only OVA on alum protected significantly compared to non-immunized mice ($p=0.0420$).

To rule out any role of T cells in the observed protection against anaphylaxis, serum from IgE-sensitized BALB/c mice was transferred to immunized athymic nude mice twenty hours before the OVA challenge (**Fig. 6C**). The maximum average temperature drop following challenge was 5.3 °C in mice immunized with peptide-loaded liposomes, and 8.5 °C in non-immunized mice (**Fig. 6D**).

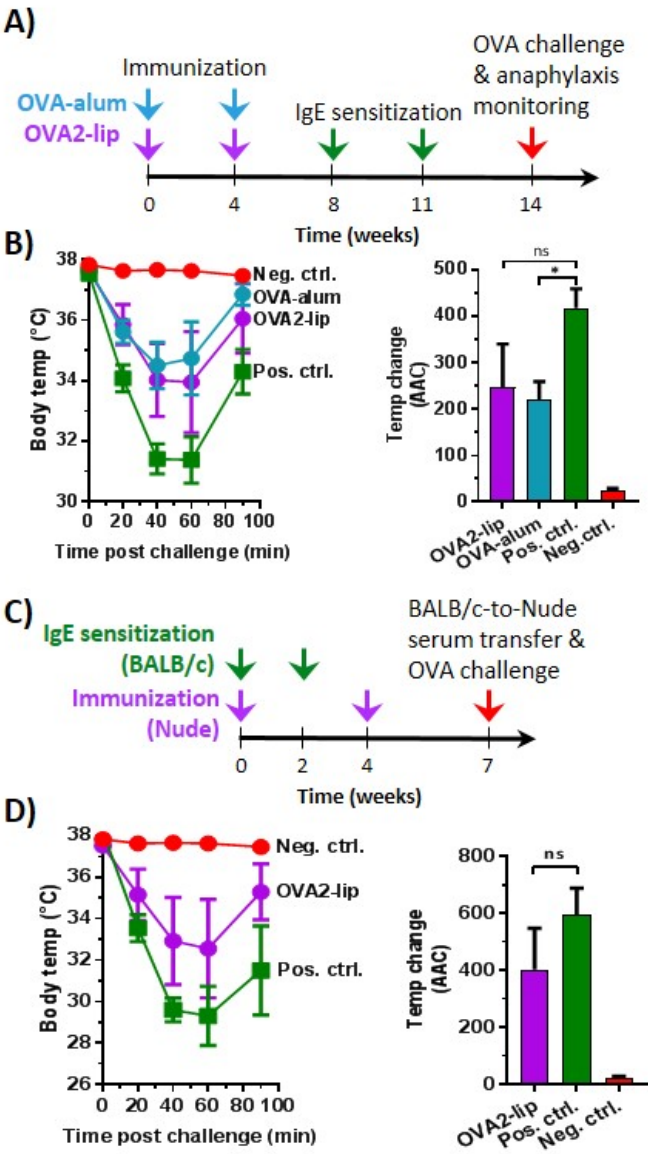

Figure 6: Immunization with peptide- and MPLA-loaded liposomes reduces susceptibility to sensitization independent on T cells. (**A**) BALB/c mice were immunized twice (weeks 0 and 4) with OVA2 peptide- and MPLA-loaded liposomes or OVA protein on alum. In weeks 8 and 11, the mice were subject to sensitization by s.c injections of OVA protein on alum. An i.p. challenge with soluble OVA was performed in week 14. (**B**) The body temperature upon challenge was monitored as a measure for allergic anaphylaxis, and the integrated area above curve (AAC) for the body-temperature curves was calculated using pre-challenge temperature as baseline. Results are expressed as mean + SEM (n = 5 per group). * p≤ 0.05 calculated by Kruskal-Wallis test. (**C**) Nude mice were immunized with OVA2 liposomes and boosted 4 weeks later. In parallel, BALB/c mice were sensitized with two s.c injections of 100 µg OVA on alum in weeks 0 and 2. In week 7, BALB/c mice were bled and sensitized serum was transferred to the immunized nude mice; naïve nude mice served as positive controls. Challenge was performed 20 hours after serum transfer and anaphylaxis tested as described above. (**D**) Body temperatures measured in nude mice challenged with OVA and the integrated area above curve (AAC) for the body-temperature curves was calculated using pre-challenge temperature as baseline. Results are expressed as mean + SEM (n = 5 per group). * p≤ 0.05 calculated by two-tailed non-parametric Mann-Whitney test.

Prophylactic immunization of mice with peptide- and MPLA-loaded liposomes reduces bacterial growth in systemic infection of Listeria monocytogenes in vivo

Since one of the advantages of immunization with T-cell independent vaccines could be the fast onset of antibody responses, one could foresee applications in pandemics, where fast and effective vaccination coverage is important. Here, mice were immunized with the OVA peptide-loaded liposomes and subsequently infected with OVA-expressing *L. monocytogenes*. Although innate immunity and T cells are considered the most important factors controlling listeriosis [232, 233], immunized mice displayed significantly diminished bacterial loads in liver (5.2-fold, p=0.0159) as compared with infected but non-immunized mice (**Fig. 7A**). A minor protective effect was also determined in the spleen. After immunization with OVA protein on alum, a partial but not significant protection was observed. At the time of infection, only OVA-specific IgG1 was detected in sera from mice immunized with OVA-alum (**Fig. 7B**). To evaluate the long-term function of peptide-induced TI antibodies, mice were immunized with OVA2 liposomes and infected 6 months later with a lethal dose of OVA-expressing *L. monocytogenes*. Significantly lower number of bacteria were observed in the spleen of immunized mice compared to non-immunized mice (p=0.0159) (**Fig. 7C**). The same, but non-significant trend was observed for the bacterial load in the liver. At the time of the infection, hence, six months after a single injection of the vaccine, positive and high levels of antigen-specific IgG1, IgG2a, IgG2b, and IgG3 were determined (**Fig.7D**). None of the serum IgG subclasses correlated directly with bacterial load in organs.

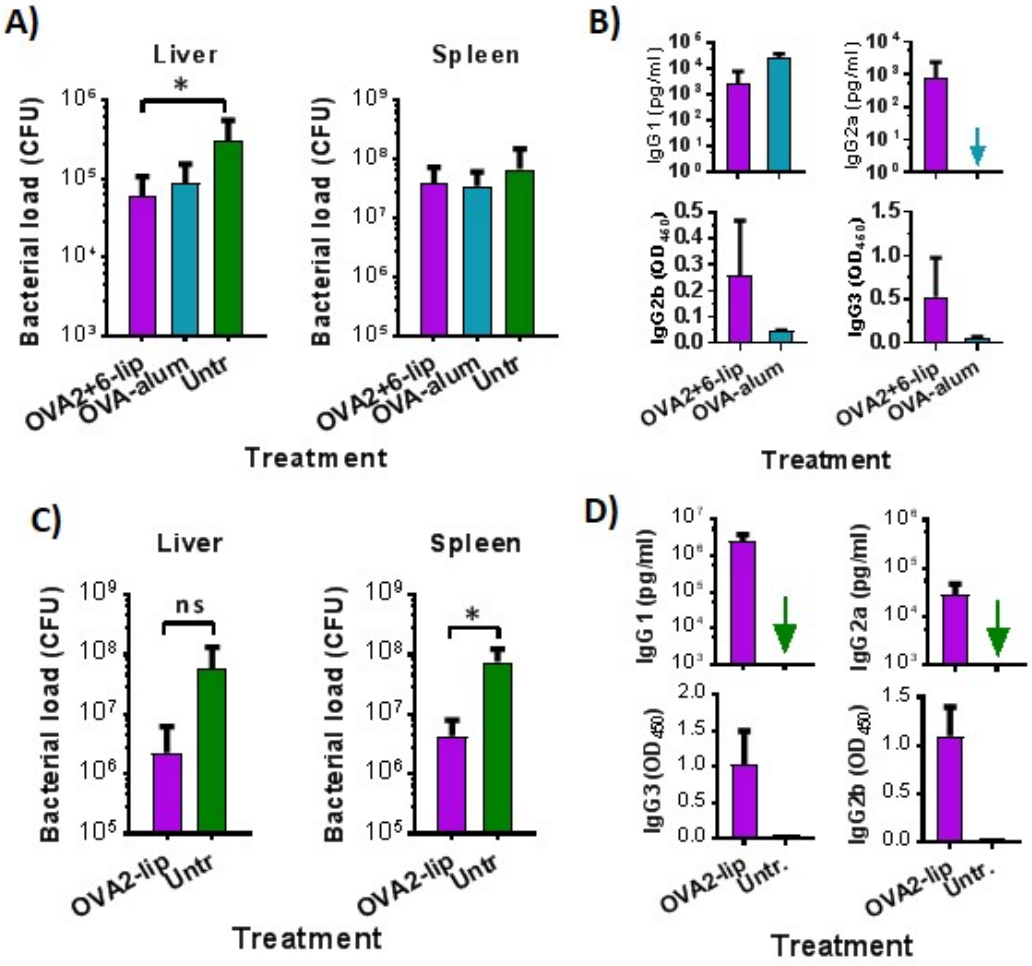

Figure 7: Immunization with peptide- and MPLA-loaded liposomes reduces bacterial load in organs after Listeria infection. (**A-B**) Mice were immunized with a mixture of 10 µg OVA2 and OVA6 peptide liposomes or 100 µg OVA on alum seven days before infection with 10.000 CFU *L. monocytogenes* expressing OVA. (**A**) Bacterial load in spleen and liver as well as (**B**) OVA-specific antibodies in blood were determined 48 h after infection and one day before infection, respectively. (**C-D**) Mice were immunized in weeks 0, 4 and 25 with 10 µg OVA2 peptide liposomes and infected with 10.000 CFU Listeria in week 28. (**C**) Bacterial load in spleen and liver as well as (**D**) OVA-specific antibodies in blood were determined 48 h after infection and one day before infection, respectively. Results are expressed as mean + SD (n = 5 per group). * p≤ 0.05 calculated by two-tailed non-parametric Mann-Whitney test.

Discussion

In the current study, we demonstrate that immunization with liposomes loaded with peptide antigens and the TLR4 ligand MPLA, stimulated B cells fully independent on T cells. Moreover, the long-lasting IgG responses could be boosted months after the primary immunization. To our knowledge, this is the first description of *in vivo* recall memory B-cell responses after immunization with TI peptide antigens. The switch to all IgG subclasses took place within four days of immunization in athymic nude mice and in mice deficient of αβ and γδ T cells or of MHC class II. The antibody responses displayed characteristics of both TI-1 and TI-2, since depending on TLR4 signaling through MyD88 and BTK signal transmission. GCs formed three days after immunization and were still visible after three weeks, and IgG affinity maturation was evidenced by the increased avidity over time. The TI antibodies were found to be functional using murine models of allergy and infection.

TI antigens do not require CD4 T_H cells for elicitation of antibody responses. Some TI-1 antigens, such as LPS, can stimulate B cells to proliferate *in vitro* through TLR signaling, resulting in antigen non-specific antibodies. However, efficient stimulation of antigen-specific antibody responses to TI-1 antigens require concomitant stimulation of BCRs and TLRs, both *in vitro* and *in vivo* [52]. In contrast, TI-2 antigens elicit antigen-specific antibody responses through extensive cross-linking of BCRs [63]. The simultaneous stimulation of 10-20 BCRs, can be sufficient to activate the B cell in the absence of T_H cells or other co-stimulatory signals [234, 235]. By these means, repetitive polysaccharide antigens on gram-positive bacteria can elicit potent IgM responses but limited class switch in the absence of T_H cells [10, 61]. Viral envelopes also have highly organized protein structures and can elicit TI IgM and IgG antibodies, especially IgG3. However, antibody responses to non-replicating protein antigens, as in subunit vaccines, are characterized by IgM antibodies with no class switch to IgG, IgA or IgE in the absence of T_H cells [202, 236-239].

Simultaneous activation of TLRs and BCR further strengthens the B-cell activation and result in SYK-mediated activation of the NF-κB pathways [114, 202, 240, 241]. MPLA is a detoxified derivative of lipid A and LPS, a TLR4 ligand [242], and it is the only adjuvant next to aluminum salts approved for use in humans. Our study demonstrates that a high density of peptides and MPLA on liposome surfaces provides strong enough signals to activate B cells directly and to induce immunoglobulin class switch to all IgG subclasses. The broad distribution of IgG subclasses in response to the peptide-loaded liposomes is atypical for TI antibody responses, indicating a thus far unknown mechanism. The TI antibody response requires that peptide and MPLA are incorporated into the surface of the same individual liposomes ([228] and data not shown), suggesting that peptide and MPLA bind the same B

cell and that antigen-presenting cells (APCs) are not involved. Moreover, the limiting parameter for T-cell independency is peptide density rather than peptide dose.

TD murine immune responses usually involve all IgG subclasses, but their relative abundance varies as a result of the prevailing cytokine environment, which again is affected by interactions of many cell populations within the lymphoid tissues [87]. We found that peptide- and MPLA-loaded liposomes stimulated a balanced IgG response with expression of all subclasses, while the TD antigen mostly elicited IgG1 production. Using liposomes loaded with MPLA and a 15mer amyloid-β peptide [228], we were the first to demonstrate IgG class switch with a non-replicating peptide antigen in the absence of T cells. However, while liposomes with amyloid-β triggered low levels of IgG2a, IgG2c, and IgG1, liposomes with OVA peptides triggered high IgG2a and IgG1 levels.

The antibody response induced by immunization with the peptide-loaded liposomes displayed characteristics of both TI-1 and TI-2 responses. Type 1 TI (TI-1) antigens require TLR signaling to induce antibody responses [9]. The adjuvant MPLA can bind to either TLR4 or CD14, both known LPS receptors. In the current study, antibody production was abrogated in TLR4 deficient mice, and we already showed that antibody responses and IgG class switch was independent of CD14 co-stimulation [228]. The antibody responses were unaffected in TRIF-, but abolished in MyD88-deficient mice, as observed for the two peptides (OVA2 and OVA6) tested in MyD88- and TRIF-deficient mice. In contrast, liposomes loaded with the amyloid-β 1-15 peptide stimulated antibody responses in a TRIF-dependent and MyD88-independent manner [228]. It is currently unclear what dictates whether TLR4 utilizes the MyD88 or TRIF signaling pathway. In macrophages, CD14-independent signaling by TLR4 was dependent on MyD88, whereas CD14-dependent signaling activated TRIF [243]. More recently, initial activation on the plasma membrane in macrophages and DCs was found to result in MyD88 signaling followed by CD14-dependent endocytosis of TLRs [244], where TRIF signaling subsequently ensues. Surface expression of CD14 is limited to myeloid cells, but soluble CD14 can be released and at high concentrations substitute for TLR-associated CD14 on other cell types, e.g. B cells [243]. Despite B cells being activated through TLRs and playing an essential role in responses to pathogens, most studies on the two TLR4 signaling pathways have been performed in DCs and macrophages. In B cells, LPS and its immunogenic moiety lipid A have been found to stimulate TLR4 signaling through MyD88 [103, 245, 246]. In the presence of IL-4, TRIF signaling can become more important [245, 247, 248]. TLR signaling stimulated with TD antigens was primarily TRIF dependent [248], indicating that T-cell involvement may play a role in the shift to TRIF signaling. However, it remains to be shown whether B cells can internalize TLR4 for TRIF signaling and if there is a difference between B cells and myeloid cells in the

localization of the TLR4 [245]. Hence, MyD88 and TRIF pathways may play different roles in regulating TLR4-induced immune responses in B cells [113].

Type-2 TI (TI-2) antigens require intracellular signaling through BTK, following cross-linking of BCRs [9]. With the peptide-loaded liposomes, IgM antibody production as well as IgG2a class switch were surprisingly normal in BTK-deficient mice, while no class switch to IgG1, IgG2b, and IgG3 was observed. Hence, BTK may play distinct roles in class switch to different subclasses.

Although, TI antigens are not expected to cause GC formation, somatic hypermutation, or B-cell memory, immunization with peptide-loaded liposomes enabled T-cell independent GC formation and increased IgG avidity. A few studies have reported short-lived GCs in the absence of T cells, but the studies used nitrophenyl conjugated to polysaccharide (NP-Ficoll) as antigen in TCR$\beta\delta^{-/-}$ mice [51] and transgenic mice with high numbers of NP-specific B cells [52, 53]. We were the first to report IgG class switch and GC formation using an amyloid-β-derived peptide and MPLA on liposomes [228]. GCs formed in TCR-deficient mice independent of CD28 and CD40L ligation. In the current study with OVA-derived peptides, we demonstrate that the GCs were T-cell independent and had a long lifespan after a single immunization whereas NP-Ficoll-induced GCs were comparably short-lived [52]. In our study with OVA peptide-loaded liposomes, TI-associated GCs were marginally smaller and less frequent than GCs formed in response to control immunization with TD antigens.

In TD-associated GCs, T cells are not needed until the stage of centrocyte selection in the light zone, after which a proportion of positively selected centrocytes cycles back into the dark zone, as centroblasts, for further somatic hypermutation. The centroblasts likely undergo a finite number of divisions before they mature into centrocytes, in which case the pool of centroblasts can run out unless it is renewed [55]. This could explain the sudden termination of the NP-Ficoll-induced TI GC reaction as it collapses by massive B-cell apoptosis a few hours after the dark zone and light zone form, due to lack of positive selection and renewal of the centrocyte pool in the absence of T_{FH} cells [53]. Interestingly, we found peptide-induced GCs were still visible on day seven in TCR-deficient mice [228], as long as 20 days in BALB/c mice, and 10 days in athymic nude mice. More work will be needed to find out why GCs survive in the absence of positive selection by antigen-specific T cells, but NF-κB has been shown to control GC maintenance and differentiation in TD responses [249], and it is activated by TLR4 signaling through MyD88 [98, 112]. GCs induced by non-peptide TI antigens are non-functional with only limited mutations in transgenic quasimonoclonal mice with unusually strong B-cell signaling [53], but not in wild type mice [54]. In contrast, we found increased avidity of each individual IgG subclass after immunization with peptide-loaded liposomes. Interestingly, a booster immunization with the

alum-adsorbed and T-cell dependent protein vaccines, but not the liposomes, increased the antibody avidity further, indicating that affinity maturation is completed earlier for the TI antigen. The biological function of the antibodies was demonstrated *in vivo* in murine model of allergic anaphylaxis and *Listeria* infection. The affinity maturation of B cells can take place both in GCs and, in the absence T cells, in the extrafollicular space [27, 250, 251]. In fact, T-cell independent expression of AID, an enzyme involved in somatic hypermutation, was observed during B-cell development in a process involving both BCRs and TLRs [252]. This result supports a mechanism of action by which peptide- and MPLA-loaded liposomes, which also induces expression of AID [228], may enable somatic hypermutation and affinity maturation independent of CD4 T_H cells.

Antibody responses to the peptide- and MPLA-loaded liposomes have a fast onset and long duration with secondary injections giving a memory-like boost in serum IgG. Two days after immunization, IgM was detected in sera and the response peaks two days later, in line with responses to the TI antigen heat-killed *Streptococcus pneumoniae* [4]. While the latter study did not detect immunoglobulin class switch, we found IgG class switch starting on day 3. In contrast, IgG class switch after immunization with TD antigens does not start earlier than on day 6 [71]. Moreover, IgG levels remained high for months after immunization with liposomes, and booster immunizations after 7 or 14 weeks demonstrated a memory recall response as IgG levels increased beyond the levels determined after the primary immunization. To our knowledge, this has never been described for TI peptide antigens. Memory B cells have been shown to be inducible with haptenated TI antigens, however, recall antibody responses could only be obtained when primed B cells were adoptively transferred to naïve, T-cell deficient recipients, before secondary antigen exposure [10, 56, 58-61]. Direct memory response has not been shown in the animal it was originally generated in [8], and the TI B-cell memory has been found to be regulated through negative feedback by antigen-specific antibodies [59, 61]. A marginal increase in IgG antibody-forming cells was reported upon secondary immunization with NP coupled to αCD180 antibody in CD40 deficient mice ten weeks after the primary injection [253]. Furthermore, the IgG antibody responses was shown to be partially dependent on T cells, as the titers were severely impaired in CD40- and TCR-deficient mice and hardly detectable in MHC II-deficient mice [253]. The lack of B-cell recall responses in the absence of T cells raises the question how B cells and antibodies can be protective upon secondary infection if they are so tightly controlled. In responses to TD antigens, a second memory compartment of long-lived bone-marrow plasma cells is generated in addition to memory B cells [65, 66]. The bone marrow plasma cells have been believed to be strictly T-cell dependent and require formation of GCs [67]. Yet, the TI-2 antigen S. pneumoniae capsular polysaccharide, generated long-lived bone marrow plasma cells secreting protective IgM and IgG [62].

Nonetheless, the plasma cells generated by TD and TI antigens are functionally different, suggesting that the memory plasma cell compartment is heterogeneous [62]. It is unclear when the memory compartment comes into play upon secondary exposure to antigen, and the contribution of TI memory B cells and TI memory plasma cells may vary depending on antigen type and persistence as well as the route of antigen entry.

Vaccination is the most effective measure to prevent against infections [115]. Protective immune responses often correlate with the affinity of neutralizing antigen-specific antibodies, for which reason the key to success is the generation of memory B cells [121, 122, 180]. In certain situations, it would however be important to stimulate B-cell responses without involvement of T cells. Patients suffering from T-cell deficiencies, such as HIV, SCID, DiGeorge or Wiskott-Aldrich syndromes, or the elderly could benefit from TI vaccines [254, 255]. Moreover, a TI vaccine could also be used in vaccination against self-antigens such as amyloid-β, where it is important to avoid elicitation of cytotoxic T cells that may cause unwanted autoimmune tissue damage [256]. TI vaccines may also prove useful in diseases where rapid immune protection is needed, but where conventional TD vaccines are not recommended, justified, or efficient, e.g. for the protection against pathogens during epidemic infectious outbreaks or against bioterrorism [8, 117, 257]. Vaccine strategies without T-cell involvement have not yet been explored, because TI peptide antigens have been believed to lack immunoglobulin class switch and memory B cells [61]. In light of the growing body of evidence on protective B-cell memory forming without CD4 T_H cells, the true potential of TI vaccines should be revisited [8, 61, 62, 228]. Here, the peptide- and MPLA-loaded liposomes may serve as a useful tool for investigating further the characteristics of TI memory and the potential of such immune responses in disease and vaccination.

Chapter 3

On the feasibility of T-cell independent allergen immunotherapy

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Introduction

Type-I allergic sensitization is the process by which the body produces IgE as response to environmental and otherwise inert proteins contained in grass- and tree pollen, animal dander, dust mites and more. The sensitization depends on T_H2 differentiation of allergen-specific CD4 helper T (T_H) cells that promote class switch to IgE [157]. While drugs such as antihistamines and corticosteroids control peaks of rhino conjunctival symptoms and asthma, allergen immunotherapy (AIT) is the only disease-modifying treatment with long-term effects [144]. AIT aims at stimulating a shift from the T_H2 response underlying the allergy, towards a counteracting T_H1 response. AIT also stimulates the differentiation and production of CD25- and Foxp3-positive CD4 regulatory T cells (T_{REGs}), which play an important role in the induction and maintenance of immune tolerance [157]. Finally, AIT leads to a re-programming of B cells to produce allergen-neutralizing antibodies, especially of IgG1 and IgG4 subclasses, and the concomitant suppression of disease-mediating IgE. IgG antibodies are believed to prevent allergic responses by binding allergen and thereby obstructing it from cross-linking IgE on effector cells [132, 141]. Eventually, these T- and B-cell responses are mechanistically considered to provide suppression of allergic inflammation and symptoms. At present, AIT can be obtained by subcutaneous (SCIT) or sublingual (SLIT) methods. Both SCIT and SCLIT require multiple administrations of allergen over years, for which reason patient compliance is challenging. Moreover, AIT, and especially SCIT, is associated with adverse allergic effects because of AIT-allergen reactivity with IgE, which in the following can cause mast cell or basophil degranulation [144, 258, 259]. AIT research is aimed at simplifying treatment regimens, increasing AIT safety, and enhancing efficacy of AIT [132].

While CD4 T cells surely influence allergen-specific immune responses and efficacy in AIT, the current study addressed the judicious question if the protective effects are not only affected by, but also depending on the contribution of T cells. This question is based on the fact that transferred antiserum can provide protection against allergic reactions in the host [259, 260]. Moreover, since effective protection against anaphylaxis must take place within seconds or minutes of the allergen exposure, e.g. as caused by bee or wasp bites, circulating allergen-specific antibodies most likely play an important effector role. However, the induction of such effector antibodies during AIT may still be strongly regulated by T cells. To test whether T cells are necessary for successful AIT, we applied an obligate T-cell independent (TI) vaccine (cf. *chapter 2*) in the AIT and studied its therapeutic potential in a mouse model of anaphylaxis.

Methods

Mice

BALB/c (BALB/cOlaHsd, H-2^{d}) and athymic nude (Hsd:AthymicNude-Fox1) mice were purchased from Envigo (Horst, the Netherlands). The mice were kept and bred under SPF conditions at the animal facility of the *Biologisches Zentrallabor*, University Hospital Zurich. Only female mice were used, and all procedures were approved by the animal Research Ethics Board and the Zurich cantonal veterinary office (license 98/2014).

Preparation of peptide liposomes

Liposomes were prepared from dimyristoyl phosphatidyl choline (DMPC), dimyristoyl phosphatidyl glycerol (DMPG), cholesterol (Solvay, Brussels, Belgium), and monophosphoryl lipid A (MPLA; Avanti Polar Lipids, Alabaster, USA) as previously described [215]. A short peptide derived from chicken ovalbumin (OVA) was used as antigen for formulation in the liposomes. The OVA peptide (OVA aa58-72; RFDKLPGFGDSIEAQ) was synthesized and flanked with two palmitoylated lysines (Lys) on each terminal side (PolyPeptide Laboratories, Strasbourg, France). The liposomes were loaded with 409 µg/ml peptide and 56 µg/ml MPLA.

Sensitization and immunotherapy

Groups of five female BALB/c mice were sensitized subcutaneously (s.c.) at the scruff of the neck with full Grade V OVA protein from Sigma-Aldrich (Buchs, Switzerland) mixed with aluminum hydroxide (Alum; Alhydrogel 2%) from Brenntag Biosector (Frederikssund, Denmark). OVA was dissolved in phosphate-buffer saline (PBS) and allowed to absorb on alum for one hour at room temperature before injection. The sensitization dose was 1 µg OVA and 1 mg alum in 100 µL PBS, and four injections were made with weekly intervals.

T-cell independent AIT was performed with doses of 10 µg OVA peptide and 1.4 µg MPLA on liposomes diluted in PBS. T-cell dependent control AIT was performed with a vaccine preparation of 10 µg OVA protein on alum. Both vaccines were administered by s.c. injections. The AIT was repeated for a total of two or four injections, the time interval between each injection being one, two, or six weeks as indicated. A group of control mice were sensitized but did not receive AIT ("No AIT"), while a group of challenge control mice were neither sensitized nor received AIT ("Untreated").

Blood was collected from tail veins of sensitized and treated mice at different time points, and the OVA-specific IgG1, IgG2a, and IgE in serum measured by means of ELISA.

Allergen challenge and analysis of systemic anaphylaxis

Three weeks after the last AIT, 20 or 25 µg OVA in 100 µl PBS was given intraperitoneally (i.p.). This challenge causes an anaphylactic reaction in sensitized mice, and the anaphylaxis can be detected as a drop in body temperature, as monitored using a digital rectal thermometer (Thermalert TH-5 with a RET-3 probe, Physitemp, Huron, NJ).

Measurement of OVA-specific antibody responses by ELISA

OVA-specific IgG1, IgG2a, and IgE antibody responses were determined by a sandwich ELISA as previously described [226]. Briefly, the plates were coated with 4 µg/ml OVA for IgG subclass detection and with 3 µg/ml anti-mouse IgE (AbD Serotec, Düsseldorf, Germany) for detection of IgE. OVA-specific IgE was detected using in-house biotin-conjugated OVA, while IgG subclasses were detected with biotin-conjugated goat anti-mouse IgG subclass-specific antibodies (Abcam, Cambridge, United Kingdom). All plates were developed with streptavidin-conjugated horseradish peroxidase (HRP) and with TMB substrate (eBioscience, San Diego, CA, now Thermo Fischer). Reaction was stopped with 2N sulphuric acid and absorbance read at 450 nm on a BioTek Instruments plate reader (Winooski, VT). The serum concentration of OVA-specific antibodies was calculated against standards for IgG1, IgG2a (BioLegend, San Diego, CA) and IgE (AbD Serotec).

Statistical analysis

The statistical analysis were performed using the software GraphPad Prism 7.02 software. The non-parametric data were analyzed by Kruskal-Wallis analysis for multiple comparisons or by Mann-Whitney analysis for analysis of two groups. The significance level was set at 0.05.

Results

The T-cell dependency of the two AIT regimes were first monitored in T-cell deficient nude mice. The TI vaccine with OVA-peptide and MPLA loaded on liposomes induced IgG production in both BALB/c and nude mice, while the T-cell dependent (TD) vaccine based on alum-adsorbed OVA protein only stimulated IgG antibodies in BALB/c mice (**Fig. 1**).

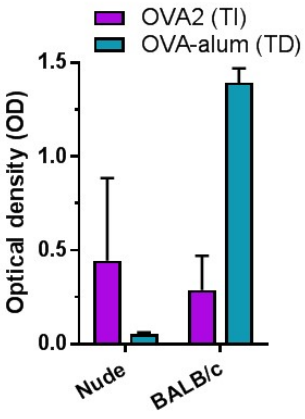

Figure 1: OVA-specific IgG1 antibodies in nude and BALB/c mice measured by ELISA. The mice received 4 weekly s.c injections of 10 µg OVA peptide-loaded liposomes or 30 µg OVA on alum. Blood was collected 2 weeks after last injection. Results are expressed as mean + SD (n = 3 per group).

Wild type BALB/c mice were then sensitized and treated with two AIT injections either weekly or fortnightly as illustrated in **Figure 2A**. Sensitized mice, that did not receive AIT showed a hypothermic reaction in response to the systemic allergen challenge (**Fig. 2B**; No AIT). Mice treated with the liposomal TI vaccine showed a similar hypothermic reaction to that observed in the otherwise untreated allergic mice. In contrast, AIT with the TD vaccine based on OVA and alum provided partial protection as seen by reduced hypothermia compared with sensitized control mice (fortnightly AIT, p=0.013) and mice treated with the TI vaccine (weekly AIT, p=0.009). Fortnightly AIT with the TI vaccine reduced the hypothermic reactions significantly more than weekly treatment (p=0.016). Neither weekly nor fortnightly AIT with the TI vaccine had significant effects on the measured hypothermic reactions.

Sensitization induced anti-OVA IgG1, IgG2a, and IgE antibodies, as illustrated for sera collected before the first AIT session (**Fig. 2C**, open bars). During the following five weeks (week 11), the serum levels of OVA-specific IgG but not IgE, increased further in the sensitized control mice (**Fig. 2C**, filled bars). AIT with the TI vaccine did not generate a significant boost in serum IgG1 and IgG2a when compared to control mice, and IgE levels remained comparable to those measured in sera of sensitized control mice. In contrast, AIT with the TD vaccine resulted in an increase in IgG1, IgG2a, and IgE.

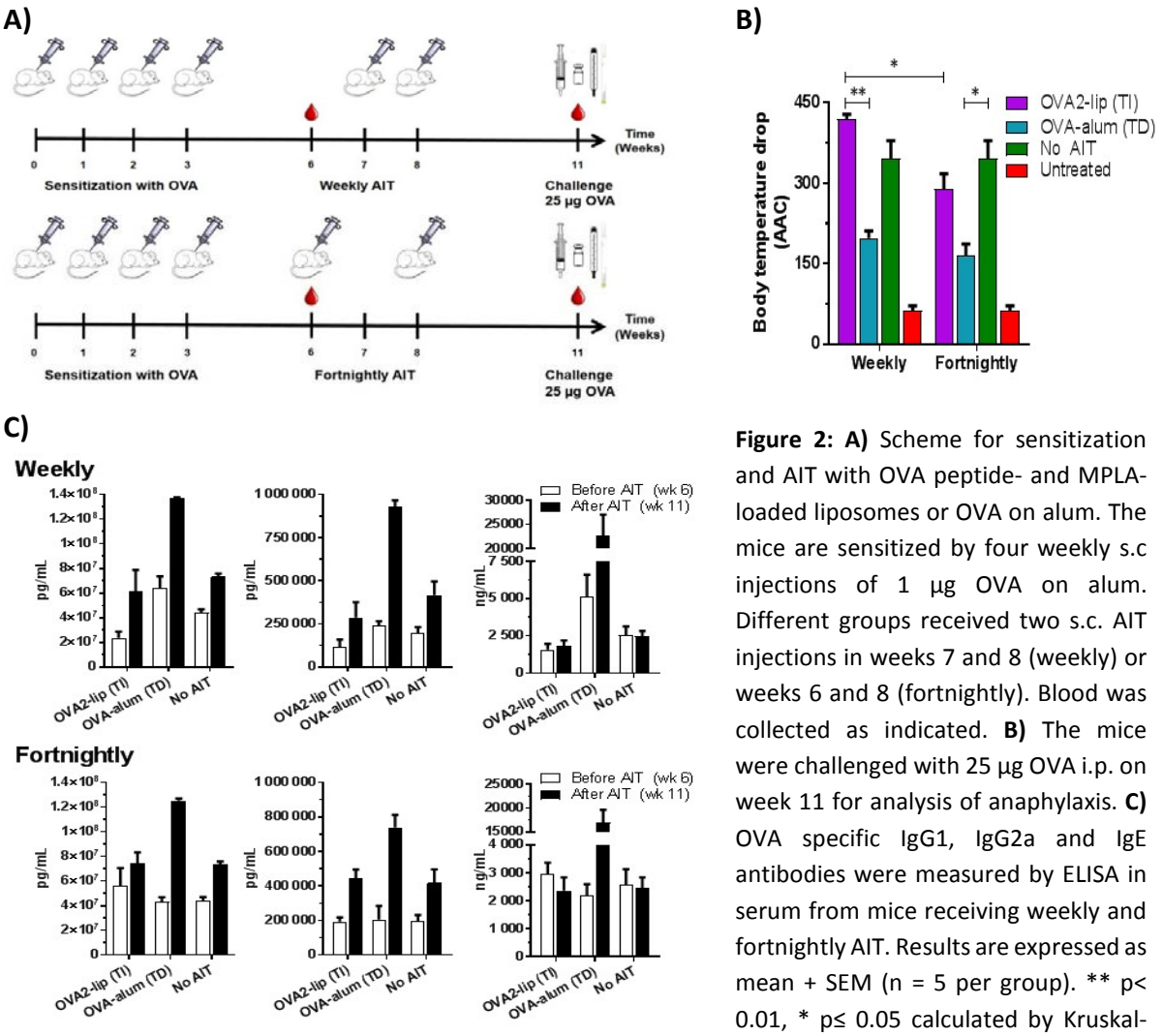

Figure 2: A) Scheme for sensitization and AIT with OVA peptide- and MPLA-loaded liposomes or OVA on alum. The mice are sensitized by four weekly s.c injections of 1 µg OVA on alum. Different groups received two s.c. AIT injections in weeks 7 and 8 (weekly) or weeks 6 and 8 (fortnightly). Blood was collected as indicated. **B)** The mice were challenged with 25 µg OVA i.p. on week 11 for analysis of anaphylaxis. **C)** OVA specific IgG1, IgG2a and IgE antibodies were measured by ELISA in serum from mice receiving weekly and fortnightly AIT. Results are expressed as mean + SEM (n = 5 per group). ** p< 0.01, * p≤ 0.05 calculated by Kruskal-Wallis and Mann-Whitney when comparing treatment intervals.

Next, we tested whether longer AIT time intervals or higher numbers of AIT injections could mediate protection against anaphylaxis upon systemic challenge. Mice were sensitized as described above, but the AIT interval was increased to six weeks and compared to four fortnightly AIT injections (**Fig. 3A**). AIT with the liposomal TI vaccine produced no protection against allergic anaphylaxis, as the measured hypothermic reactions did not differ from that measured in mice that received no AIT (**Fig. 3B**). In contrast, AIT with the TD vaccine produced symptom relief and significantly less hypothermia as compared to the sensitized control mice (6-weekly AIT, p= 0.024). However, whether the AIT was administered with two- or six-week intervals had no significant effect on the measured hypothermic reactions.

Sensitization induced anti-OVA IgG1, IgG2a, and IgE antibodies, as illustrated for sera collected before the first AIT (**Fig. 3C**, open bars). During the following nine weeks (week 15), the serum levels of OVA-specific IgG, but not those of IgE, increased further in the sensitized control mice receiving no AIT (**Fig.**

3C, filled bars). AIT with the liposomal TI vaccine did not generate a significant boost in serum IgG1 and IgG2a when compared to control mice that did not receive AIT, and the IgE levels remained comparable to those measured in sera of sensitized control mice. In contrast, AIT with the TD vaccine resulted in an increase in IgG1, IgG2a, and IgE (**Fig. 3C**).

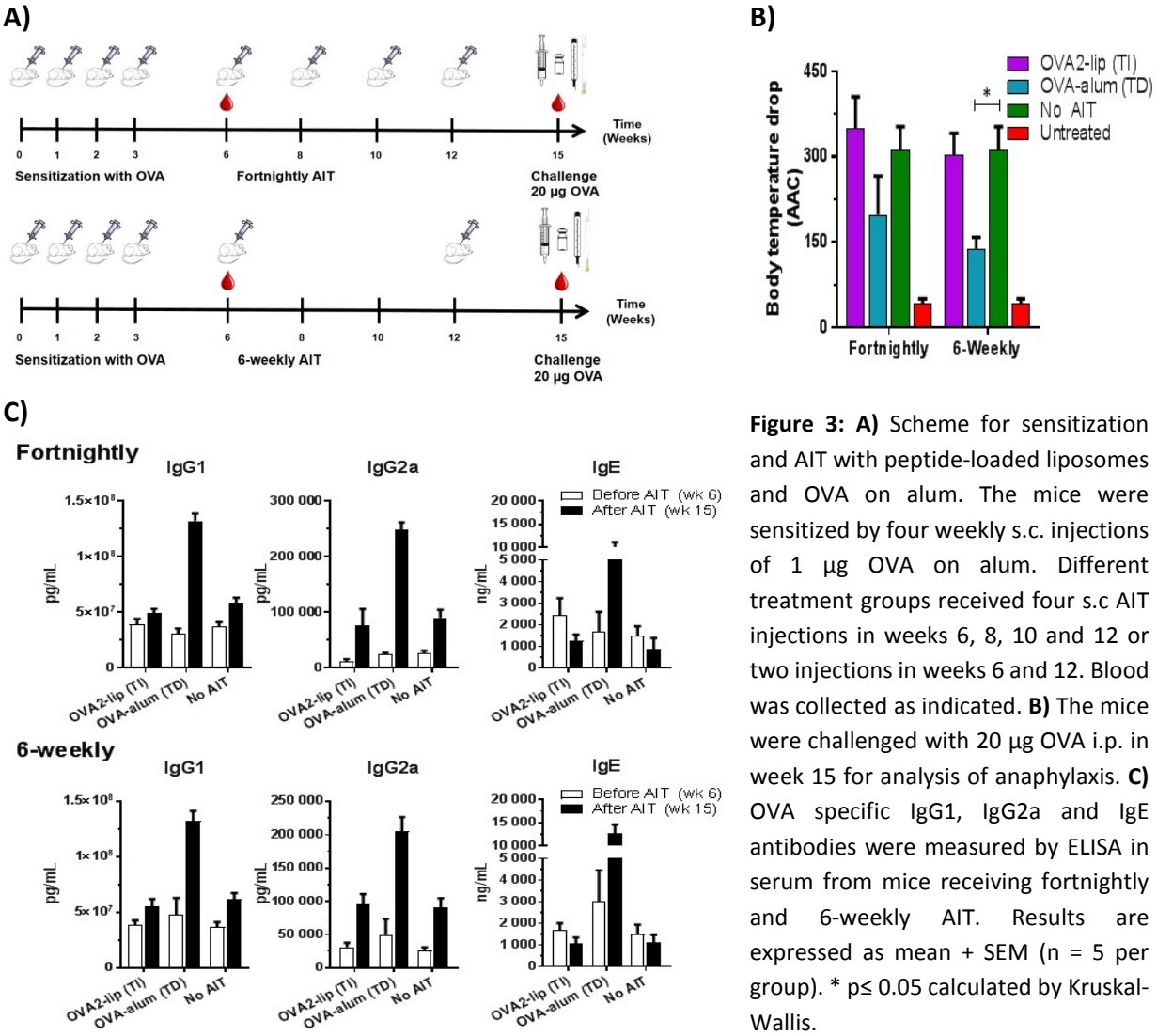

Figure 3: A) Scheme for sensitization and AIT with peptide-loaded liposomes and OVA on alum. The mice were sensitized by four weekly s.c. injections of 1 µg OVA on alum. Different treatment groups received four s.c AIT injections in weeks 6, 8, 10 and 12 or two injections in weeks 6 and 12. Blood was collected as indicated. **B)** The mice were challenged with 20 µg OVA i.p. in week 15 for analysis of anaphylaxis. **C)** OVA specific IgG1, IgG2a and IgE antibodies were measured by ELISA in serum from mice receiving fortnightly and 6-weekly AIT. Results are expressed as mean + SEM (n = 5 per group). * p≤ 0.05 calculated by Kruskal-Wallis.

Since AIT with T-cell independent peptide-loaded liposomes did not protect OVA-sensitized mice against allergic anaphylaxis, we tested whether the lack of protection was due to non-efficient AIT or due to sensitization potential of the liposome-based treatment itself. Mice received a single injection of the liposomal TI vaccine or the alum-based TD vaccine, the doses being 6 µg OVA2 peptide and 100 µg OVA protein, respectively. While a single injection of the TD vaccine stimulated production of OVA-specific IgE antibodies, the TI vaccine stimulated no OVA-specific IgE antibodies (**Fig. 4A**). When challenged with a systemic injection of 20 µg adjuvant-free OVA (**Fig. 4B**), mice that received the TD

vaccine OVA on alum reacted with significantly stronger hypothermic reactions than mice that received the TI vaccine based on peptide-loaded liposomes (p=0.001). The latter mice showed no temperature reaction (**Fig 4B**) or other monitored anaphylactic symptoms such as piloerection, hunched posture, or apathy (not shown).

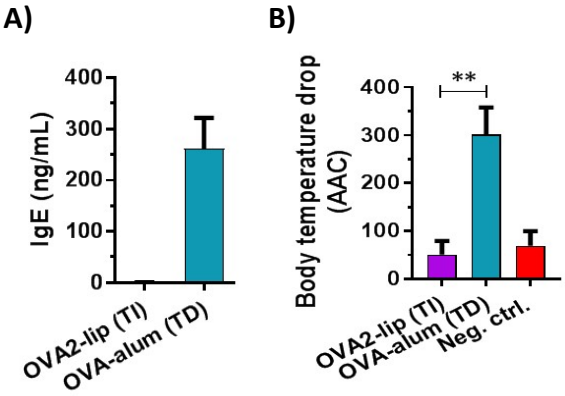

Figure 4: A) OVA specific IgE antibodies measured by ELISA in serum from mice collected before challenge. **B)** Changes in body temperature after mice were sensitized once with peptide-loaded liposomes or 100 µg OVA on alum and challenged with 20 µg OVA i.p. two weeks later for analysis of anaphylaxis. Results are expressed as mean + SEM (n = 5 per group). ** p< 0.01 calculated by two-tailed non-parametric Mann-Whitney U test, comparing OVA2-lip and OVA-alum.

Discussion

In the current study, we investigated whether T cells were required for successful AIT in a mouse model of anaphylaxis. Sensitized mice were treated with a T-cell independent (TI) AIT regime of OVA peptide- and MPLA-loaded liposomes or with a T-cell dependent (TD) AIT regime based on OVA protein adsorbed on alum. Despite stimulating OVA-specific IgG responses, AIT with the TI vaccine provided no protection against anaphylactic reactions in OVA-sensitized mice. In contrast, AIT with the TD vaccine resulted in a partial relief from hypothermia and other monitored anaphylactic reactions after the systemic challenge.

The serum concentration of IgG4 in humans is an important biomarker for successful AIT [261, 262], and IgG2a is considered to have similar effects in mice as IgG4 has in humans [263]. Indeed, AIT with the TD vaccine boosted serum IgG2a and gave rise to the strongest relief from allergic symptoms upon the systemic challenge for induction of anaphylaxis. Despite the protective efficacy of AIT with the TD vaccine, this AIT regime also stimulated a strong increase in the production of OVA-specific IgE when compared with the serum levels determined after sensitization alone. However, production of IgE has also been reported to increase early in AIT in humans and the serum levels of IgE does not directly correlate with the clinical improvement [132, 141, 166].

At current, it is not understood why the TI liposomal vaccine, which very efficiently produced high titers of allergen-specific IgG1, IgG2a, IgG2b, and IgG3 antibodies in mice (as shown in *chapter 2*), did not protect against systemic anaphylaxis in sensitized mice. This lack of protective efficacy was not a

result of an IgE-stimulating and sensitizing potential of the vaccine itself, since its administration did not cause induction of allergen-specific IgE or anaphylactic reactions such as hypothermia after a systemic allergen provocation. Another explanation for the lack of protective capacity of the TI vaccines could be insufficient polyclonality of the induced antibody response. In response to a protein antigen, numerous different epitopes on the protein can stimulate production of a diverse repertoire of polyclonal antibodies. In contrast, the TI vaccine used in the current study consists of a single short peptide, giving a much smaller repertoire of antibodies. Increased polyclonality of the TI antibody response may be achieved by attaching different antigen-derived peptides on to the liposomes or by administration of different liposomal preparations, each with a different antigen peptide attached. Such vaccines would allow for a better evaluation of whether the functional difference is due to insufficient polyclonality of the antibody response or the difference in AIT efficacy is due to direct or indirect contribution of T cells. Finally, induction of T-cell responses could be required for successful AIT. T_{REGs} are important in inducing immune tolerance in AIT and such tolerance not only suppresses IgE antibodies and increases IgG. In addition, T_{REGs} suppress cells such as antigen-presenting cells, mast cells, basophils and eosinophils as well as interacting with resident tissue cells [132]. To what extent T cells convey protection or contribute to protection of AIT has been subject to numerous studies [260, 264-266]. Transfer of CD4 T cells from immunized mice provides partial protection from anaphylactic reaction after challenge in sensitized mice [260]. Furthermore, transfer of CD4 T cells expressing CD25, or T_{REGs}, gave more relief from allergic symptoms than did transfer of the CD25 negative population of CD4 T cells. However, transfer of whole serum from the immunized mice resulted in nearly complete protection. In another study, depletion of T_{REGs} either before or after AIT partially reversed the effects of AIT in a mouse model of allergic asthma [264], indicating that T_{REGs} are in part required for the induction of tolerance in AIT, but that the T-cell contribution in AIT may vary in the different manifestations of allergy.

The current study also suggested that longer intervals between AIT sessions might be beneficial for the outcome of the therapy. When using the TI vaccine, we found that fortnightly AIT produced better outcome than did weekly injections. A similar trend was seen for the TD vaccine but the difference was not statistically significant. These results are in line with previous reports by us, showing that longer intervals between AIT injections improve treatment efficacy [267]. Moreover, general recommendations for administration of vaccines include an interval of at least four weeks, and vaccination is considered invalid if the recommended injection interval is shortened [268-271]. As persistent high doses of antigen may inhibit B cells, longer intervals allow efficient formation of memory B cells as well as activation of high affinity T cells [272].

Allergy can manifest with a wide variety of symptoms, such as rhino-conjunctivitis, asthma, anaphylaxis, skin rash and hives, and during the elicitation phase of the allergic reactions, the relative contribution of potentially protective immune functions varies. Anaphylaxis is perhaps the clinical manifestation where we expect allergen-specific IgG antibodies to have the strongest immediate protective effect. In the current study, the murine model of anaphylaxis was therefore used to evaluate the protective efficacy of allergen-specific IgG generated after AIT with either a TD vaccine or a TI vaccine. AIT with TI peptide- and MPLA-loaded liposomes did not protect from anaphylactic reactions in sensitized mice in the experimental setup tested, while TD AIT resulted in partial symptom relief. However, in this study, serum levels of antibodies did not increase after AIT with the TI vaccine, making it difficult to make the conclusion that antibodies alone are not sufficient for successful AIT. In order to better compare the relative effects of T and B cells, the polyclonality of the TI antibody response would need to be improved. Moreover, the immunoglobulin isotype and IgG subclass distributions as well as their serum concentrations and antigen binding affinities, are all confounders that complicate a comparison of TD and TI vaccines at the level of mechanism of action. In any case, the strictly T-cell independent peptide- and MPLA-loaded liposomal formulation could serve as a novel tool for the analysis of the relative contribution of T and B cells in AIT.

Chapter 4

Dosing intervals in intralymphatic immunotherapy

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Allergen-specific subcutaneous (SCIT) as well as sublingual (SLIT) immunotherapy both confer long-term protection against the highly prevalent IgE-mediated allergies, such as allergic rhino-conjunctivitis and asthma. Since both SCIT and SLIT are both very laborious and time-consuming treatments, we and other have over the last years investigated whether the immunotherapy could be enhanced by so-called intralymphatic immunotherapy (ILIT), which aims at improving treatment efficacy and safety and to reduce treatment duration, thereby making allergen immunotherapy (AIT) more patient friendly. In the original studies made by us [273, 274] and by Cardell et al [275], ILIT was administered three times with four week intervals. In 2013, Witten et al. questioned the efficacy of ILIT with grass pollen, based on the results of a clinical study of their in 38 adult hay-fever patients [276]. While promising immunological changes such as increased IgG4 and Treg responses were observed, they concluded that the clinical outcome was at odds with that measured by us in 165 hay fever and 20 cat-dander allergic patients as well as in Cardell's study with 28 hay-fever patients. The protocols in these studies differed in one major point. While our and Cardell's studies used a dosing schedule of 4 weeks, Witten's study utilised a shorter dosing schedule of 2 weeks. We chose the longer time intervals based on general vaccine recommendations as they allow build-up of successive waves of allergen-specific immune responses with efficient memory B-cell formation and affinity maturation without antibody interference. Moreover, CD4 T cells are competing for antigen and that higher affinity T cells have a competitive advantage [277]. Of note, no FDA-approved childhood vaccine has a shorter time interval than 4 weeks, and the official guidelines consider a vaccination "invalid" if the time interval is shortened to less than the recommended minimum.

Comparably less is known about the role of time intervals in AIT, and especially in newer methods such as ILIT. To address this question experimentally, a pre-clinical test in mice was run to investigate the effects of varying ILIT dosing intervals on certain characteristics of the antibody response. Groups of five female BALB/c mice were sensitised with 1 µg ovalbumin (OVA) adsorbed to aluminium hydroxide by four weekly subcutaneous (s.c.) injections. Three weeks later, they received ILIT injections with 10 µg OVA into the inguinal lymph nodes [156]. The ILIT was repeated for a total of three injections, the time interval between each injection being one, two, or four week intervals (**Fig.1A**). ILIT preparations with OVA were made with aluminium hydroxide [156, 226] as this was the adjuvant also used in the clinical ILIT trials [273-276]. Blood was collected from tail veins at different time points during the study. OVA-specific IgG_1, IgG_{2a}, and IgE in serum as well as the OVA-specific affinity of IgG were measured at different time points by means of ELISA.

Sensitisation caused only a weak induction of anti-OVA IgG_1 a barely measurable induction of IgG_{2a}, but a clear IgE sero conversion (**Fig. 1B-D**; week 0 versus week 5). Blood IgG_1 and IgG_{2a} levels

significantly increased in all groups following ILIT, which started on week 6, indicating an effective immunotherapy, independent on the time interval between the ILIT sessions. At the end of the study, 14 weeks after the first of three ILIT injections, the 4-week interval ILIT produced the highest measured IgG_1 and IgG_{2a} levels, as compared to pre-ILIT antibody levels (**Fig. 1E-F**). While the IgG_1 levels did not show correlation between dosing intervals and endpoint antibody levels, the IgG_{2a} levels increased with greater time intervals between the ILIT injections ($p=0.0355$ by Kruskal-Wallis). The former is in line with previous reports that show that IgG_1 is a less sensitive marker for effective immunotherapy in mice than IgG_{2a} [156]. The kinetics of IgG_1 antibody production after ILIT showed that 1-week ILIT intervals produced a faster IgG_1 increase than 2- and 4-week intervals, but that the different ILIT protocols later plateaued at comparable serum levels of IgG_1 (**Fig. 1B**). The dynamics of IgG_{2a} antibody production over time was less dependent on dosing intervals. IgG_{2a} levels after ILIT with 4-week intervals rose as fast as after ILIT with shorter intervals, but reached higher endpoints levels of IgG_{2a} (**Fig. 1C**). IgE production also increased in all groups following ILIT, but the rise followed the same pattern and reached the same endpoint antibody levels independent on the ILIT dosing interval (**Fig. 1D and 1G**).

In addition to the titre of antibodies, the affinity of antibodies is an important characteristic for assessing the immunological changes following AIT. Therefore, the OVA-specific affinity of total IgG and IgG_{2a} was measured using the Martineau ELISA method [278]. A clear increase in both IgG_{2a} and total IgG affinity was observed following ILIT and as measured using the Martineau competition ELISA, with the biggest rise in IgG_{2a} affinity after 4-weekly ILIT (3.4-fold) followed by 2-weekly (2.8-fold) and weekly (2.4-fold) ILIT compared to pre-ILIT affinity values (data not shown). The competition ELISA also allowed calculation of the antigen-antibody affinity constant (Ka), which describes the strength of the antibody-antigen binding. **Table 1** shows the total IgG Ka for sera collected one week after the last ILIT or at the study endpoint (10-16 weeks after last ILIT). A clear positive effect of longer ILIT-intervals was observed as the Ka increased from 1.0×10^{-6} M^{-1} after weekly ILIT, to the 3-fold (2-week ILIT) and 13-fold (4-week ILIT) as measured 1 week after the last of three ILIT injections. Interestingly, when the affinity was measured at the end of the study (week 24), the 2-weeks ILIT produced high-affinity antibodies, comparable to those produced during the 4-week ILIT intervals. Hence, the kinetics of the affinity maturation seems to be affected by the dosing interval, which again may affect the outcome of functional tests performed at different time points after ILIT. The increased affinity with longer intervals is probably a result of limited antigen so that only B cells bearing progressively higher affinity antibody may continue to be stimulated. In contrast, persistently high doses of antigen have an

inhibitory effect on the B cells that are most efficient in trapping antigen. Hence, with low doses and/or long dosing intervals, a selective tolerance of high affinity B cells is obtained [272].

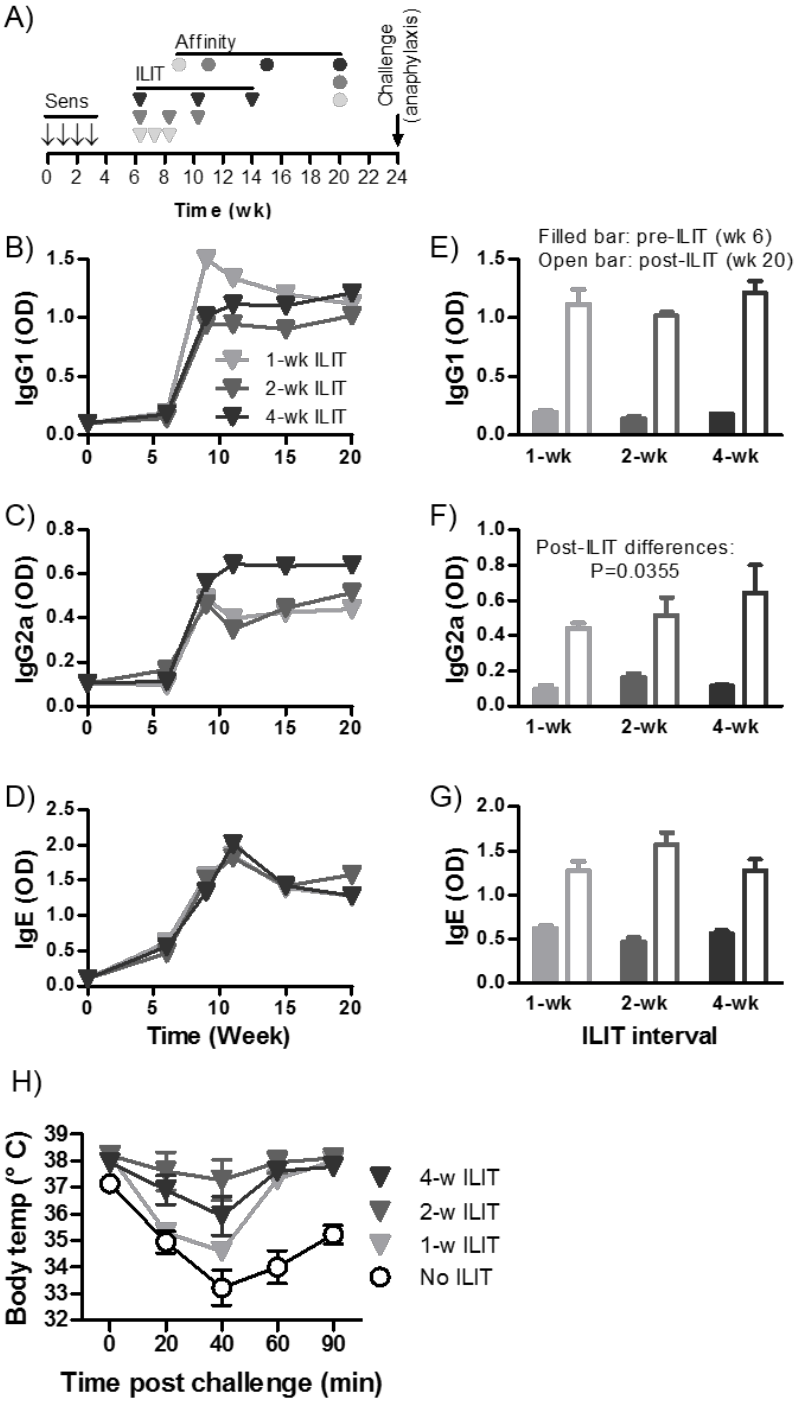

Figure 1. (**A**) Timeline for sensitization, ILIT (triangles), affinity antibody measurements (circles), and anaphylaxis test. Mice were sensitized to OVA by weekly s.c. injections and subsequently treated in three ILIT sessions with 1, 2, or 4 weeks dosing intervals. IgG_1 (**B, E**), IgG_{2a} (**C, F**) and IgE (**D, G**) antibody responses were measured during 20 weeks, with the last observation carried forward to impute any missing values for the 2-week group. (**H**) On week 24, all ILIT mice as well as sensitization control mice were challenged with OVA in saline (i.p.) and the anaphylaxis was measured as changes in body temperature. The different shades of grey in line, symbols, and bars represent the different dosing intervals, the darker the grey, the longer the interval.

Table 1. Affinity constant (Ka) of OVA-specific total IgG antibodies as measured one week post last ILIT and at the end of the study (16, 14 and 10 weeks after last ILIT).

	Affinity constant Ka	
Interval	**1 wk post ILIT** ($\times 10^{-6}$ M^{-1})	**Study endpoint** ($\times 10^{-6}$ M^{-1})
1 week	1.07	2.96
2 weeks	3.04	12.3
4 weeks	12.8	10.8

Additionally, when the protection against experimental anaphylaxis was determined upon challenge with a high-dose of allergen in saline given intraperitoneally, differences were observed between the different time intervals ($P<0.01$ by 2-way ANOVA). Briefly, all ILIT-treated mice experienced symptom relief as measured by change in body temperature and as compared to sensitised controls that did not receive ILIT (**Fig. 1H**). Partial protection was measured for 1-week ILIT, while more or almost complete protection was measured for 2-week ILIT and 4-week ILIT. No significance difference was calculated for the two latter.

In contrast to previous reports on 4-weekly ILIT by us [273, 274] and others [275], Malling's randomised, double-blind, placebo controlled ILIT study showed no significant clinical efficacy of weekly or bi-weekly injections based on seasonal hay fever symptoms and medication scores (SMS), despite them being reduced [276]. More recently, Patterson et al. have reported positive effect of 4-weekly ILIT in adolescents and young adults (personal communication), However, intradermal tests (IDT) readings and grass pollen skin prick tests (SPT) showed significant reduction in both the treatment and the placebo groups, but with no statistically significant difference between groups. Serum concentrations of IgG_4, an important biomarker for successful AIT [261], increased after grass pollen ILIT, with a slightly stronger increase after bi-weekly ILIT than after weekly ILIT. This correlates with our findings that longer ILIT intervals results in higher serum IgG_{2a} levels, an antibody subclass considered important to murine AIT similar to IgG_4 in human AIT [226]. Grass-pollen-specific serum IgE levels increased after both bi-weekly and weekly ILIT in the human study by Malling et al. [276], similar to what observed in the present murine study, also with no dependency on the ILIT interval.

In conclusion, ILIT caused a marked rise in IgG_1 and IgG_{2a} antibodies independent on the dosing interval. However, there was a tendency of stronger antigen-specific IgG antibody responses, especially that of IgG_{2a}, with higher affinity after longer ILIT dosing intervals. The same effect was observed in a vaccination protocol with one, two, four or eight week immunisation intervals in naïve mice (unpublished data). The present study was conducted in mice and the results can, of course, not be

directly translated to ILIT in humans. However, the data substantiate the claim that the efficacy of the ILIT and perhaps other forms of AIT may depend on the time interval between administrations of the therapeutic allergen preparations, similar to that of prophylactic vaccinations against infectious diseases in human. We are convinced that correct dosing intervals can improve AIT and that dosing interval should be considered and further investigated in future human clinical studies. However, in order to collect comparable data from different studies, it is pivotal to apply comparable primary outcomes and to estimate clinical efficacy with standardised and generally approved methods. Such results are expected to be available in 1-2 years, as the results of several clinical trials will be released, all performing ILIT by 3 injections with 4 week intervals. Sang Min Lee and co-workers at Gachon University Gil Medical Center are doing animal dander and dust mite ILIT (NCT02301884), Søren Helbo SH Skaarup and co-workers at Aarhus University Hospital are doing grass pollen ILIT (NCT02255604), Lars-Olof Cardell and co-workers at Karolinska Institutet are doing birch and grass pollen ILIT, and Amber Patterson and co-workers at Columbus Nationwide Children's Hospital, are performing grass pollen ILIT in 15-24 years old (NCT01982474).

Chapter 5

Cytosolic delivery of liposomal vaccines by means of concomitant photosensitisation of phagosomes

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Abstract

One of the greatest pharmaceutical challenges in vaccinology is the delivery of antigens to cytosol of antigen-presenting cells (APCs) in order to allow the stimulation of major histocompatibility complex (MHC) class I-restricted CD8+ T-cell responses, which may act on intracellular infections or cancer. Recently, we described a novel method for cytotoxic T-lymphocyte (CTL) vaccination by combining antigens with photosensitiser and light for cytosolic antigen delivery. The goal of the current project was to test this immunisation method with particle-based formulations. Liposomes were prepared from dipalmitoyl phosphatidylcholine and cholesterol, and the antigen ovalbumin (OVA) or the photosensitiser tetraphenyl chlorine disulfonate (TPCS2a) was separately encapsulated. C57BL/6 mice were immunised intradermally with OVA liposomes or a combination of OVA and TPCS2a liposomes, and light was applied next day for activation of the photosensitiser resulting in cytosolic release of antigen from phagosomes. Immune responses were tested both after a prime only regime and after a prime-boost scheme with a repeat immunisation two weeks post priming. Antigen-specific CD8+ T-cell responses and antibody responses were analysed ex vivo by flow cytometry and ELISA methods. The physicochemical stability of liposomes upon storage and light exposure was analysed in vitro. Immunisation with both TPCS2a- and OVA-containing liposomes greatly improved CD8+ T-cell responses as compared to immunisation without TPCS2a and as measured by proliferation in vivo and cytokine secretion ex vivo. In contrast, OVA-specific antibody responses (IgG1 and IgG2c) were reduced after immunisation with TPCS2a-containing liposomes. The liposomal formulation protected the photosensitiser from light-induced inactivation during storage. In conclusion, the photosensitiser TPCS2a was successfully formulated in liposomes and enabled a shift from MHC class II to MHC class I antigen processing and presentation for stimulation of strong CD8+ T-cell responses. Therefore, photosensitive particulate vaccines may have the potential to add to current vaccine practice a new method of vaccination that, as opposed to current vaccines, can stimulate strong CD8+ T-cell responses.

Introduction

Current vaccines typically contain colloidal aluminium salts as adjuvants for antigens and elicit efficient antibody responses, but only weak T-cell responses, especially those mediated by cytotoxic $CD8^+$ T lymphocytes (CTLs). For this reason, current vaccines are typically prophylactic against diseases caused by extracellular microbes whose replication can be prevented or their toxins neutralised by immunoglobulins. However, intracellular pathogens such as Mycobacteria, Legionella, Brucella, Shigella and Leishmania as well as viruses, especially non-cytolytic viruses, may be better controlled by cytotoxic $CD8^+$ T lymphocytes capable to recognise and kill infected cells. Similarly, cancer vaccines require strong activation of the CD8 arm of immunity, as suggested by studies with subunit vaccines, peptide-bearing autologous dendritic cells (DCs), or by adoptive transfer of tumour-specific $CD8^+$ lymphocytes [208, 279-286]. When $CD8^+$ T-cell-inducing vaccination has so far been poorly efficient, this is ascribed, in part, to inefficient delivery of antigen to the cytosol of antigen presenting cells (APCs), a prerequisite for accessing the MHC class I pathway of antigen presentation. For $CD8^+$ T-cell-inducing vaccination, functionally intact antigen must be transported across several barriers to reach intracellular sites of action. Both the plasma and phagosomal membranes impede antigen from cytosolic MHC class I processing and presentation [287]. As the efficiency of $CD8^+$ T-cell induction furthermore depends on the frequency and duration of antigen presentation by functionally activated APCs [288-292], it is likewise important that high doses of vaccine antigen is translocated to the cytosol of APCs [293]. Antigens of conventional vaccines typically end up in the MHC class II pathway of antigen presentation after maturation and fusion of phagosomes and lysosomes [294-296], a process that leads to $CD4^+$ T-helper cell stimulation and antibody production and only minute amounts of antigen leaking to cytosol for stimulation of MHC class I-restricted CTLs.

A number of strategies have been investigated to enhance cellular uptake and cytosolic delivery of vaccine antigens. Membrane translocating peptides such the HIV-derived TAT, the HSV-derived VP22, or protamine [297-300] can shuffle proteins across plasma membranes. Moreover, as cellular membranes possess a net negative charge, cationic liposomes or cationic nano- and microparticles have also been used to improve cellular targeting of vaccines [301, 302]. Since vaccines and especially vaccine particles normally end up in phagosomes, several strategies have also been implemented to trigger antigen release from the phagosomes into the cytosol. For instance, pH-sensitive liposomes undergo destabilization and acquire membrane fusogenic properties as pH drops in maturing phagosomes, a process that may lead to cytosolic antigen release [303]. The combination of liposomes and immune response modifiers or adjuvants has also been suggested for the stimulation of $CD8^+$ T-cell responses [304, 305]. By the same token, phagosomolytic proteins such as listeriolysin and

perfringolysin can be recombinantly integrated in antigens or in vaccine particles in order to facilitate cytosolic escape of antigen by lysis of the phagosomal membrane [306-308]. Another antigen delivery system that has been shown to stimulate CTLs is ISCOMs or ISCOMATRIX®, which is composed of nano-sized micelles of saponin, cholesterol, and phospholipids, and whose adjuvancy is in part mediated by its fast and efficient draining to lymph nodes [309-311]. As viruses naturally infect the cytosol of cells, cytosolic targeting with viral vehicles such as adeno and Vaccinia (MVA) viruses has been widely used [312, 313]. Finally, the success of subunit vaccine developments is hardly thinkable without the inclusion of immune-response modifiers or adjuvants that provide pathogen-associated (PAMP) and danger-associated (DAMP) molecular patterns and signals activating the innate immune system [314-317]

Recently, we demonstrated that photosensitisers can be utilised to target soluble antigens to the cytosol for stimulation of MHC class I-restricted $CD8^+$ T-cell responses [318-320]. Briefly, we hypothesized that a photosensitiser with affinity to cell membrane would translocate to the endosomal membrane upon its endocytosis and uptake in APCs. If the photosensitiser is co-delivered with the antigen, subsequent light activation of the photosensitiser would cause the generation of free radicals, which again would cause damage of the endosomal membrane. As a consequence, the endosome would become leaky and thereby release antigen into the cytosol. In the cytosol, the antigen would have access to the MHC class I for presentation to and activation of CTLs; **Figure 1** illustrates the concept this so-called PCI (photochemical internalization)-based immunisation. In the present study, we applied this technology to a liposome-based formulation in a murine model of immunisation. This study showed that combined administration of antigen and photosensitiser in liposomes can mediate efficient antigen translocation to the cytosol and thereby enhance $CD8^+$ T-cell stimulation.

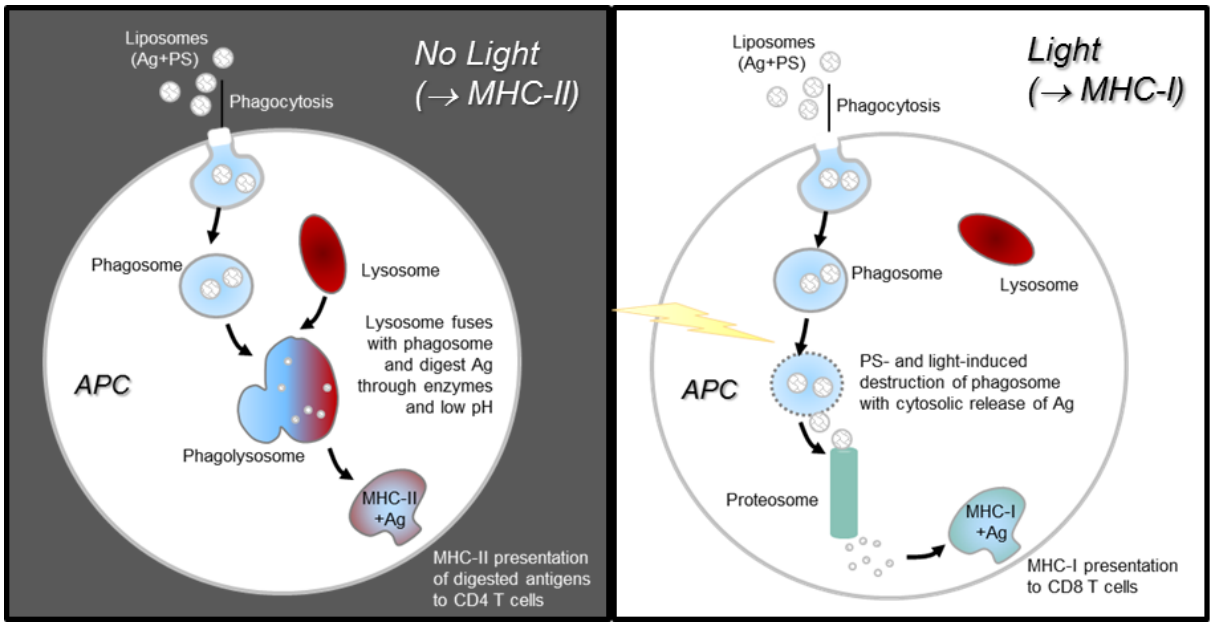

Figure 1. Illustration of PCI-based vaccination with liposomes. Liposome vaccines are taken up by antigen-presenting cells (APCs) by phagocytosis. Typically, the phagosomes mature and fuse with lysosomes for MHC-II-restricted presentation of antigen (Ag) peptides to $CD4^+$ T cells. This is also the default presentation pathway of vaccines containing photosensitiser (PS), when no light is applied (left scheme). If light is applied (right scheme), PS inside the phagosomes will be activated and produce reactive oxygen species that may cause disruption of the phagosomal membrane. The now leaky phagosomes release Ag to cytosol, where the proteasomes can digest Ag to MHC-I-binding peptides that consequently can be presented to $CD8^+$ T cells.

Methods

Animals

Female C57BL/6 mice were purchased from Harlan (Horst, The Netherlands) and used for immunisation at the age of 6-7 weeks. Rag2/OT-I mice (B6.129S6-Rag2tm1Fwa Tg(TcraTcrb)1100Mjb) from Taconic Europe (Ry, Denmark) were bred in own specific pathogen-free (SPF) facilities at the University of Zurich; $CD8^+$ T cells from Rag2/OT-I mice recognise the H-2Kb-restricted epitope SIINFEKL from ovalbumin (OVA, aa257-264). All mice were kept under SPF conditions, and the experiments performed were approved by a Swiss cantonal veterinary authority (licence 69/2012 from the Zurich Vet Office).

Materials

The photosensitiser tetraphenyl chlorine disulfonate (TPCS2a) was provided by PCI Biotech (Lysaker, Norway) and stored in the dark in a solution containing polysorbate 80, mannitol and 50 mM Tris of pH 8,5. Dipalmitoyl phosphatidylcholine (DPPC) was purchased from Lipoid (Ludwigshafen, Germany).

Cholesterol, albumin from chicken egg white (OVA; grade V), Triton X-100, RPMI 1640 medium as well as red blood cell lysis buffer were from Sigma-Aldrich (Buchs, Switzerland). The SIINFEKL peptide was synthesised by ECM microcollections (Tübingen, Germany). H-2Kb/SIINFEKL Pro 5 pentamer was bought from Proimmune (Oxford, UK). Fluoraldehyde protein assay reagent (o-phthalaldehyde, OPA) was purchased from Thermo Scientific (Reinach, Switzerland). Aluminium hydroxide (Alhydrogel adjuvant 2%) was from InvivoGen and purchased from LabForce (Muttenz, Switzerland).

Preparation of liposomes

DPPC and cholesterol were separately dissolved in chloroform to a final concentration of 100 mM each and stored at -20 °C (stock solutions). For the preparation of liposomes, the stock solutions were mixed in molar ratios of 55:45, and chloroform was slowly removed in a rotary evaporator under mild vacuum at 40 °C to obtain a homogeneous lipid film after which the lipid film was kept under vacuum overnight. The dried film was hydrated and vortexed with a solution of 800 µg/mL of TPCS2a or 200 µg/mL of OVA in 10 mM PBS of pH 8 supplemented with 0.006% polysorbate 80. The final lipid concentration was 100 mM. Complete hydration of the lipid film and liposome formation was promoted by heating the mixture in a water bath at 45 °C. The resulting multilamellar liposomes were made unilamellar by six rounds of freeze-thawing and then extruded ten times through polycarbonate membranes of 400 nm pore size (Steriltech, Kent, USA).

Characterisation of TPCS2a- and OVA-containing liposomes

After extrusion, liposomes were separated from the buffer by centrifugal filtration through Amicon® Ultra, 0.5 ml, 100k, filters (Merck Millipore, Darmstadt, Germany) for 10 min at 14000 × g. The filters were then reversed and spun for 2 min at 1000 × g for recovery of the liposomes. To determine the amount of TPCS2a inside the liposomes, the vesicles were destroyed and diluted with a 1% solution of Triton-X 100. TPCS2a concentration in the solution was measured fluorimetrically (λ_{ex} 423 nm, λ_{em} 655 nm; Infinite M200 PRO, Tecan).

For measuring the OVA content of the liposomes, the separated liposomes were destroyed and diluted with a 1% solution of Triton X-100, as described above. The OVA concentration was then measured using the OPA assay according to the manufacturer's instruction. Briefly, 200 µl OPA reagent were mixed with 20 µl of OVA solution from the destroyed liposomes, and the mixture transferred to a microtiter plate. Fluorescence intensity was measured with a plate reader (infinite® 200 PRO, Tecan, Männedorf, Switzerland) at excitation and emission wavelengths of 360 and 455 nm, respectively. OVA control standards (5-25 µg/ml) were prepared by dissolving OVA in: (i) water; (ii) Tris buffer of pH 8.5; (iii) Triton X-100 (1%); (iv) Triton X-100 (1%) with additional 55 mM DPPC; and (v) PBS of pH 8 to exclude

any interference from materials present in the liposomal preparations or used to destroy the liposomes.

Particle size and zeta potential of the liposomes were determined by dynamic light scattering and Doppler laser anemometry, respectively, using a DelsaNano C Particle Analyzer (Beckman Coulter, Krefeld, Germany). The liposomes were also assesses by cryo-scanning electron microscopy. For this, the samples were filled into a 6-mm alu-planchette and sealed with a lid. The samples were frozen in a high-pressure freezer (HPM 100, Bal-Tec/Leica, Vienna) and stored in liquid nitrogen. Vitrified samples were mounted under liquid nitrogen (-125 °C) on the cryo-holder for freeze-fracturing (BAF 060, Bal-Tec/Leica) at $1 \cdot 10^{-7}$ mbar. Coating was performed with 2 nm tungsten at an elevation angle of 45° followed by additional 2 nm under continuous elevation angle increase from 45° to 90°. Transfer to the precooled cryo-SEM (Zeiss Gemini 1530, Germany) was done under high vacuum ($<5 \times 10^{-7}$ mbar) with an air-lock shuttle and photographs were taken at -120 °C (VCT Cryostage, Bal-Tec/Leica) and inlens-SE-signals at 4 kV an acceleration voltage.

Intradermal immunisation of mice

Female C57BL/6 mice were immunised as previously described [318]. One day prior to immunisation, sex matched Rag2/OT-I spleens and lymph nodes were homogenised, the erythrocytes lysed, and the cells washed in PBS before 2×10^6 cells were administered intravenously into recipient C57BL/6 mice. The next day, the fur was shaven off the abdominal region, and 200 µl of the vaccine preparations were injected intradermally, which enables subsequent light to reach and activate the photosensitiser; deeper subcutaneous or intramuscular injections would not allow efficient light activation of the photosensitiser. The vaccine preparations administered were mixtures of 100 µl of OVA-containing liposomes with either 100 µl TPCS2a-containing liposomes, or 100 µl soluble TPCS2a (in PBS), or 100 ul PBS; mixing was done immediately before administration. The doses of OVA and TPCS2a were 2.5-25 µg and 100 µg, respectively. In some experiments, control mice received injections with the same amount of OVA adsorbed on aluminium hydroxide. After 18 h, all mice were anaesthetised by intraperitoneal injection ketamine and xylazine and placed on the LumiSource (PCI Biotech) light source for 6 min (4.86 J/cm^2) for activation of TPCS2a.

Analysis of immune responses by flow cytometry and ELISA

The frequency of antigen-specific CD8$^+$ T cells was monitored in blood and spleen by flow cytometry using H-2Kb/SIINFEKL Pro5 pentamer. Cell-surface expression of CD4, CD8, and CD44, and intracellular production of IFN-γ were analysed by flow cytometry after Fc-receptor blocking with anti-CD16/32. The intracellular staining was done after 6 h incubation at 37 °C with 0.1 µg SIINFEKL. Brefeldin A (2.5

μg/ml) was added during the last 4 h. The cells were fixed and permeabilised using BD Perm/Fix as described by BD Biosciences (Allschwil, Switzerland), and stained with anti-IFN-γ for 35 min. Pentamer staining was performed at 37 °C, all other stainings were performed at 4 °C. All steps were intercepted by washing in PBS/FCS 2%. FACS antibodies were from eBioscience (Vienna, Austria) or BD Pharmingen (BD Biosciences). Cells were analysed by FACSCanto (BD Biosciences, San Jose, USA), and the data evaluated with FlowJo 8.5.2 (Tree Star, Ashland, OR). For the cytokine secretion analysis by ELISA, 2×10^5 splenocytes were re-stimulated in round-bottom 96-well plates with 0.1 μg SIINFEKL or 10 ug OVA. Supernatants were collected after 24-72 h and analysed using cytokine ELISA ready-set-go kits for IL-2 and IFN-γ (eBioscience).

OVA-specific serum antibodies were analysed using Maxisorb ELISA plates coated with 4 μg/ml OVA. IgG1 and IgG2c were detected using biotin-conjugated rat anti-mouse antibodies from BD Pharmingen (San Diego, CA) and Abcam (Cambridge, UK), respectively, and the samples were developed using strepatavidin-conjugated HRP (BD Pharmingen) and TMB substrate (eBioscience). The optical density (OD) at a given serum dilution was measured.

Analysis of liposome and TPCS2a stability

The stability of TPCS2a in solution and liposomes was analysed in terms of its fluorescence and as a function of light exposure. Light exposure was either to natural daylight (cycles of approx. 12 h light and 12 h darkness) or to LumiSource for 1 h (48.6 J/cm^2) followed by one daylight/dark cycle of 24 h. TPCS2a formulations (approx. 1 ml) were kept in 2 ml clear glass vials with a contact surface to the LumiSource of approx. 1 cm2. Non-liposomal TPCS2a was dissolved in in either PBS with 0.006% Tween 80 or in water, while liposomal TPCS2a was kept in PBS with 0.006% Tween 80. Samples were drawn at various time points and assessed fluorimetrically in a plate reader (λex 423 nm, λem 655 nm; Infinite M200 PRO, Tecan). In addition, liposomes were also kept at 4 °C in the dark to monitor particle size and zeta potential over two weeks.

Statistics

The statistical significance of differences between two treatment groups was typically calculated using non parametric, 2-tailed Mann-Whitney U tests. When three or more groups were compared, the non-parametric Kruskal-Wallis test was applied with the Dunn's multiple comparison post hoc test. To test the effect of the photosensitiser in experiments where the vaccines were given as prime only and as prime-boost vaccines, a 2-way ANOVA was applied, assuming normally distributed data and homogeneity of variances. Significant differences were annotated with asterisks: $p<0.05$: *; $p<0.01$:

; p<0.001: *. Mean and SEM are typically shown. All calculations were done using the GraphPad Prism 5.04 software (La Jolla, CA).

Results

Characterisation of liposomal formulations

All characteristics of the liposomes are shown in **Table 1**. The liposome size and size distribution remained constant as a function of encapsulation of OVA or TPCS2a, being on average 345-351 nm with a polydispersity of 0.09-0.012. Also the zeta potential, which was -6 mV for empty liposomes, remained practically unchanged after encapsulation of OVA or TPCS2a. For the liposomes used for later immunisation, the content of OVA and TPSC2a were 19.70 or 76.17 µg per mg lipid, respectively. The encapsulation efficiency of OVA and TPCS2a were similar and in eh range of 52-55 percent.

Table 1: Characteristics of the liposomal preparations with OVA or TPCS2a.

Property	**Empty liposomes**	**OVA-liposomes**	**TPCS2a-liposomes**
Lipid concentration [mg/ml]	112	112	112
Size [nm]	347±1	351±1	345±17
Polydispersity (PDI)	0.12±0.01	0.09±0.03	0.11±0.02
Zeta potential [mV]	-6	-5	-6
Content of OVA or TPCS2a [µg/mg lipid]	-	19.70[a] 1.75[b]	76.17
Encapsulation efficiency [%]	-	52.5 ± 3.2	55.0 ± 2.1
Injected amount of lipids per dose (mg)[c]	n.a.[e]	1.269[a] 1.425[b]	1.313[d]

[a] with 25 µg OVA; [b] with 2.5 µg OVA; [c] as used for immunization; [d] with 100 µg TPCS2a; [e] not applicable, as empty liposomes have not been injected.

Impact of liposomal versus soluble photosensitiser

In a first experiment *in vivo*, mice were immunised with OVA-containing DPPC:Chol liposomes, OVA-containing liposomes admixed with soluble TPCS2a, or OVA-containing liposomes mixed with TPCS2a-containing DPPC:Chol liposomes. The dose of OVA was either 2.5 or 25 μg and that of the photosensitiser TPCS2a was fixed at 100 μg. After 6 days, mice were bled by tail bleeding, and the blood was analysed for antigen (SIINFEKL)-specific $CD8^+$ T-cell proliferation. Soluble TPCS2a did not promote MHC class I-restricted proliferation after immunisation with 2.5 μg liposomal OVA and as measured by the frequency of H-2Kb/SIINFEKL pentamer-binding $CD8^+$ T cells in flow cytometry (**Fig. 2A**). Both OVA liposomes and OVA liposomes mixed with soluble TPCS2a produced frequencies of approx. 2.5-3.0% antigen-specific $CD8^+$ T cells, relative to the total number of $CD8^+$ T cells. When mice were immunised with a mixture of OVA liposomes and TPCS2a liposomes, an approximate 4-fold increase in the frequency of specific $CD8^+$ T cells was observed. Immunisation with 25 μg OVA in liposomes did not cause increased proliferation as compared with immunisation with a tenth of the OVA dose (**Fig. 2B**). However, there was a clear tendency of increased proliferation when the OVA liposomes were mixed with soluble TPCS2a and even more so when combined with liposomal TPCS2a (**Fig. 2B**).

On day 13, the mice were euthanized, and their spleen cells restimulated *in vitro* with the CD8 epitope SIINFEKL (**Fig. 2C-D**) or with the whole OVA protein (**Fig. 2E-F**) for analysis of IFN-γ secretion. SIINFEKL-specific IFN-γ secretion was increased after immunisation with photosensitiser both at low (**Fig. 2C**) and high (**Fig. 2D**) immunisation doses of OVA. At 2.5 μg OVA, the data revealed a beneficial effect of liposomal TPCS2a over soluble TPCS2a, while at 25 μg OVA, both soluble and liposomal TPCS2a increased IFN-γ secretion as compared to cells from mice injected with liposomal OVA only. Only limited adjuvant effect of co-administered photosensitiser was observed when the cells were re-stimulated with OVA protein. This was expected, since recall with OVA protein stimulates cytokine secretion also from $CD4^+$ T cells, which are not supposed to benefit from TPCS2a.

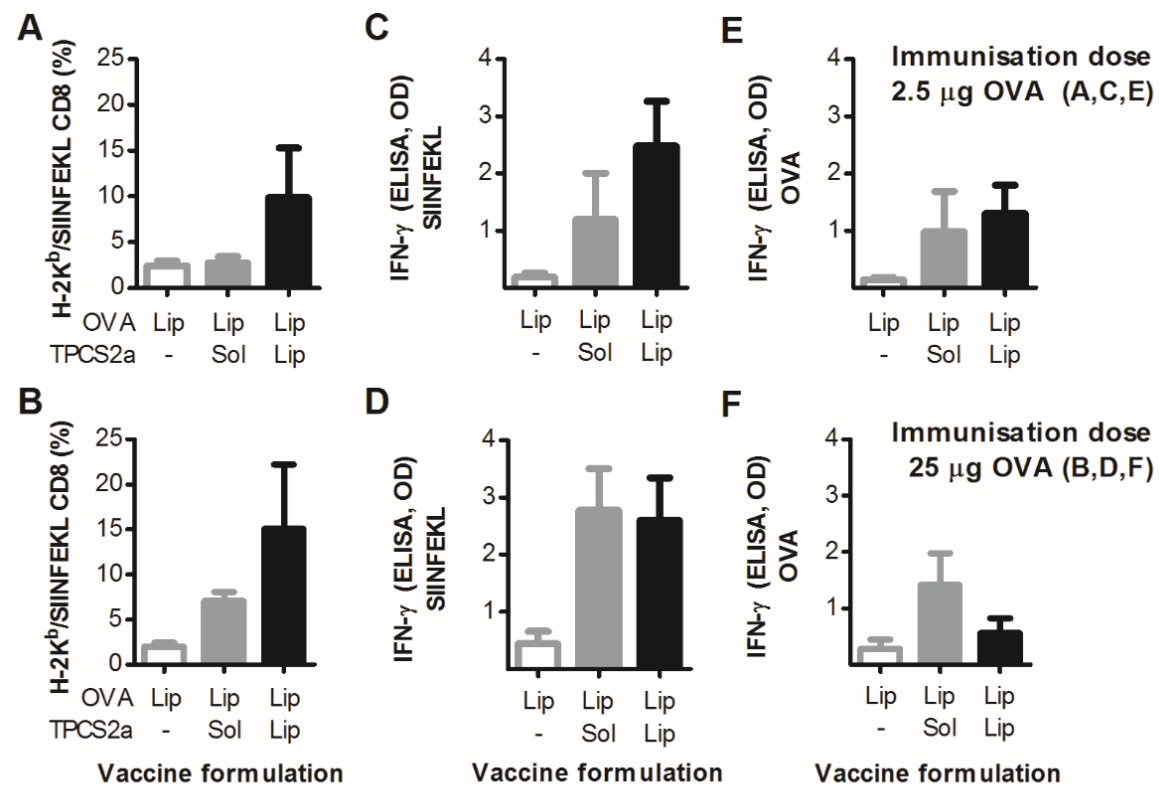

Figure 2. Immunisation with liposomes and photosensitiser. Groups of three mice were immunised with liposomes (Lip) containing OVA (open bars), with a mixture of OVA-containing liposomes and soluble photosensitiser TPCS2a (grey bars) or with a mixture of OVA-containing and TPCS2a-containing liposomes (black bars). The OVA dose was 2.5 (**A, C, E**) or 25 (**B, D, F**) μg and the TPCS2a was 100 μg (**A-F**). One day later, the mice were light-treated. On day 6 (**A-B**), mice were bled, and the frequency $CD8^+$ T cells recognising the H-2Kb-binding OVA epitope SIINFEKL was measured by pentamer staining and flow cytometry. On day 13 (**C-F**), mice were euthanized and splenocytes analysed for secretion of IFN-γ after restimulation *in vitro* with SIINFEKL (**C-D**) or OVA (**E-F**). The results represent means +SD. No statistical significances were calculated due to the low number of replicates (n=3) in this pilot experiment.

Increased antigen proliferation after immunisation with a mixture of antigen-containing liposomes and photosensitiser containing liposomes

Based on this data, further experiments were conducted with 25 μg OVA in liposomes and 100 μg TPCS2a in liposomes; both liposomal preparations were mixed within 60 min prior to the injection. In addition to an untreated control group, a control group that received OVA adsorbed on aluminium hydroxide (OVA-Alum) was included, and selected groups were given a secondary immunisation two weeks after priming (**Fig. 3A**). Proliferation of SIINFEKL-specific $CD8^+$ T cells was observed in blood 7 days after immunisation with all OVA-containing vaccine formulations, and the cells from immunised mice had an activated CD44-expressing phenotype (**Fig. 3B**). The mean frequency of SIINFEKL-specific

$CD8^+$ T cells was 1.41% for liposomal OVA, while the co-administration of TPCS2a-containing liposomes significantly enhanced the T-cell response in 8 out of 10 animals yielding a mean frequency of 5.35% SIINFEKL specific cells ($p<0.001$; **Fig. 3C**). Control immunisation with alum-adsorbed OVA resulted in an intermediate proliferation on day 7 after a single immunisation ($p<0.05$ as compared to immunisation with TPCS2a).

Secondary immunisation of antigen- and photosensitiser containing liposomes caused a strong boost of antigen specific $CD8^+$ T-cell proliferation

Fourteen days after the primary immunisation with liposomal OVA, the two liposome-treated groups of ten mice were each split into two subgroups of each five mice; one subgroup received a secondary immunisation with the same formulations as before, while the other subgroup did not receive a second injection; the OVA-Alum control group also received a booster injection. The boosting with OVA-containing and TPCS2a-containing liposomes enhanced very strongly the SIINFEKL-specific $CD8^+$ T cells; at day 22 (8 days after the booster injection), 17% of all blood $CD8^+$ T cells were SIINFEKL specific (**Fig. 3D**). On the contrary, the secondary injection with OVA-containing liposomes without TPCS2a did not significantly boost $CD8^+$ T-cell proliferation. For the mice that did not receive a booster dose, the levels of SIINFEKL-specific $CD8^+$ T cells at day 22 after priming were significantly higher when they were immunised with OVA- and TPCS2a-containing liposomes than with OVA-liposomes only ($p=0.0079$ by Mann Whitney). Immunisation with OVA adsorbed on alum did not notably boost SIINFEKL-specific $CD8^+$ T-cell numbers in blood as compared to day 7.

The longevity of the SIINFEKL-specific $CD8^+$ T-cell responses was tested four weeks after the second immunisation sessions (i.e., 6 weeks after priming). As expected, a clear retraction of T cells was observed in all groups of animals, with the frequency in mice immunised with TPCS2a-containing liposomes decreasing from 17% on day 22 to less than 1% on day 41 (**Fig. 3E**). However, the relative differences between the different immunisation groups was similar to that observed in the earlier effector phase, with a clear beneficial effect of TPCS2a-based immunisation also in the memory phase of the immune response.

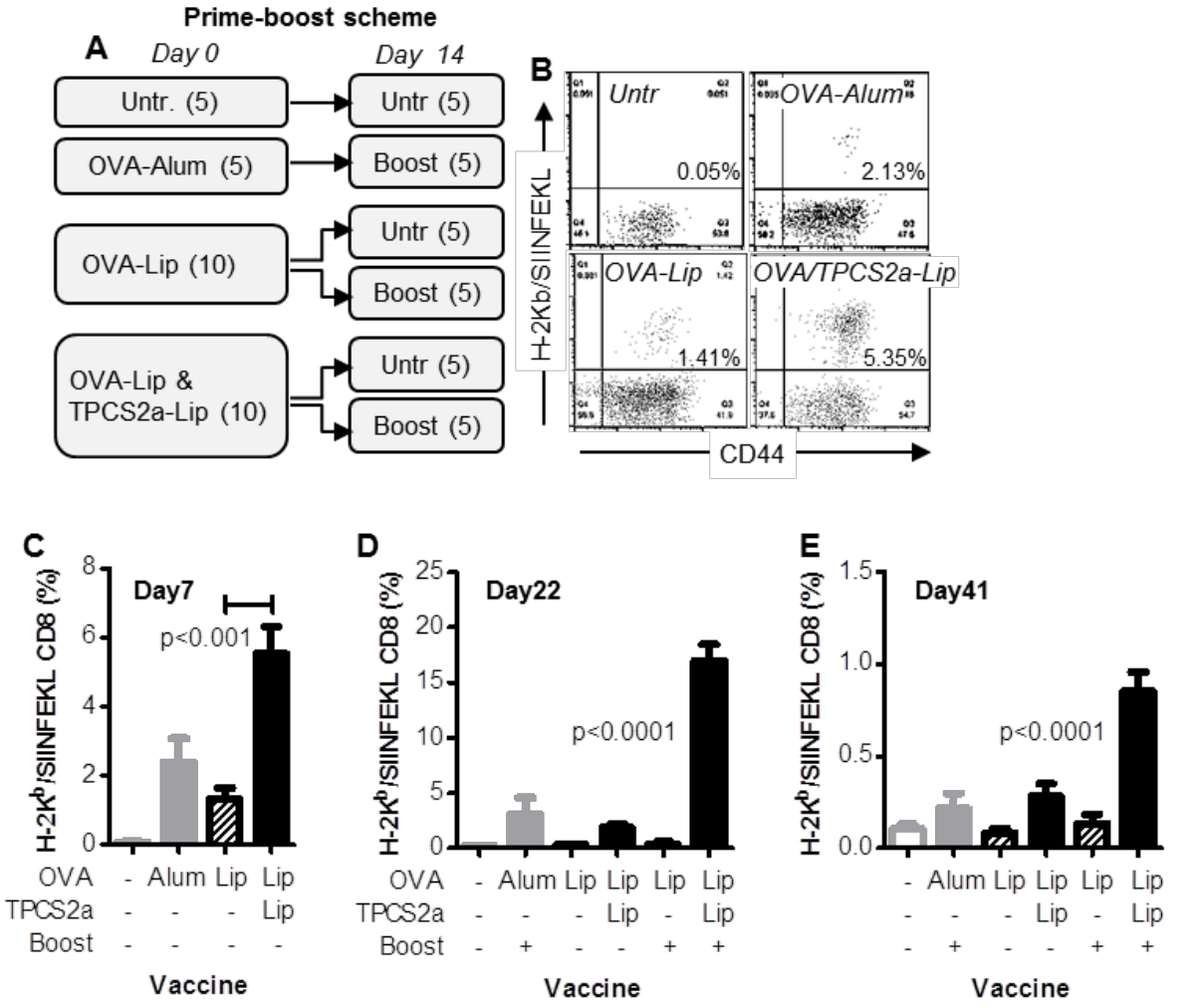

Figure 3: Antigen-specific proliferation *in vivo*. (**A**) Groups of 5-10 mice (5-10) were immunised with OVA-containing liposomes (OVA-Lip), with OVA-containing liposomes mixed with TPCS2a-containing liposomes (TPCS2a-Lip), or with OVA-adsorbed on aluminium hydroxide (OVA-Alum), while negative control mice were left untreated. OVA and TPCS2a doses were 25 µg and 100 µg, respectively, in all groups. On day 14, the immunisation was repeated for OVA-Alum, while OVA-Lip and the OVA-Lip/TPCS2a-Lip groups were split in two subgroups that were re-immunised (n=5) or not (n=5). (**B-C**) On day 7, mice were bled for analysis of SIINFEKL-specific proliferation by pentamer staining and flow cytometry. The same was measured in blood on day 22 (**D**) and spleen cells on day 41 (**E**). The data represents means + SD. Statistical significance of the difference between TPCS2a-treated and TPCS2a-untreated mice were analysed by Mann Whitney (C; p<0.001) and 2-way ANOVA (**D-E**), the parameters for the latter being TPCS2a and boost.

Immunisation with antigen- and photosensitiser containing liposomes triggered cytokine production

On days 22 (in blood; **Fig.4A-D**) and 41 (in spleens; **Fig. 4E-F**) after priming, lymphocytes were analysed for cytokine production by flow cytometry. Generally, the frequency of cytokine-producing cells was higher in mice that received booster injections than in mice that were immunised once only (**Fig. 4C-**

F). The strongest boosting effect on IFN-γ and TNF-α-producing $CD8^+$ T cells was observed for mice that received TPCS2a-containing liposomes. While the IFN-γ production was maintained or even slightly increased over 6 weeks, the TNF-α production was notably lower in the memory phase (day 41) than in the effector phase (day 22) of the immune response. Splenocytes harvested on day 41 were also restimulated *in vitro* with SIINFEKL (**Fig. 4G**) or OVA protein (**Fig. 4H**) for analysis of cytokine secretion by ELISA. Only minor effect of photosensitisation was observed with regards to SIINFEKL-specific IL-2 secretion, while a strong positive effect of TPCS2a-containing liposomes was observed for the capacity to produce IFN-γ and TNF-α (**Fig. 4G**). Indeed, the effect of photosensitisation on the production of the latter two important effector cytokines was comparatively higher than the effect on the number of cytokine-producing cells (**Fig. 4E-F**). For splenocytes re-stimulated with the whole OVA protein, no differences in IL-2 and TNF-α secretion were observed between the different treatment groups, but the results suggest some effect of photosensitisation on IFN-γ secretion (**Fig. 4H**). However, the effect was not significant and the overall secretion was approx. tenfold lower than measured in cultures of cells restimulated with SIINFEKL. Hence, the data once more indicated that TPCS2a provide a CD8-specific adjuvant effect and not a general effect on T cells.

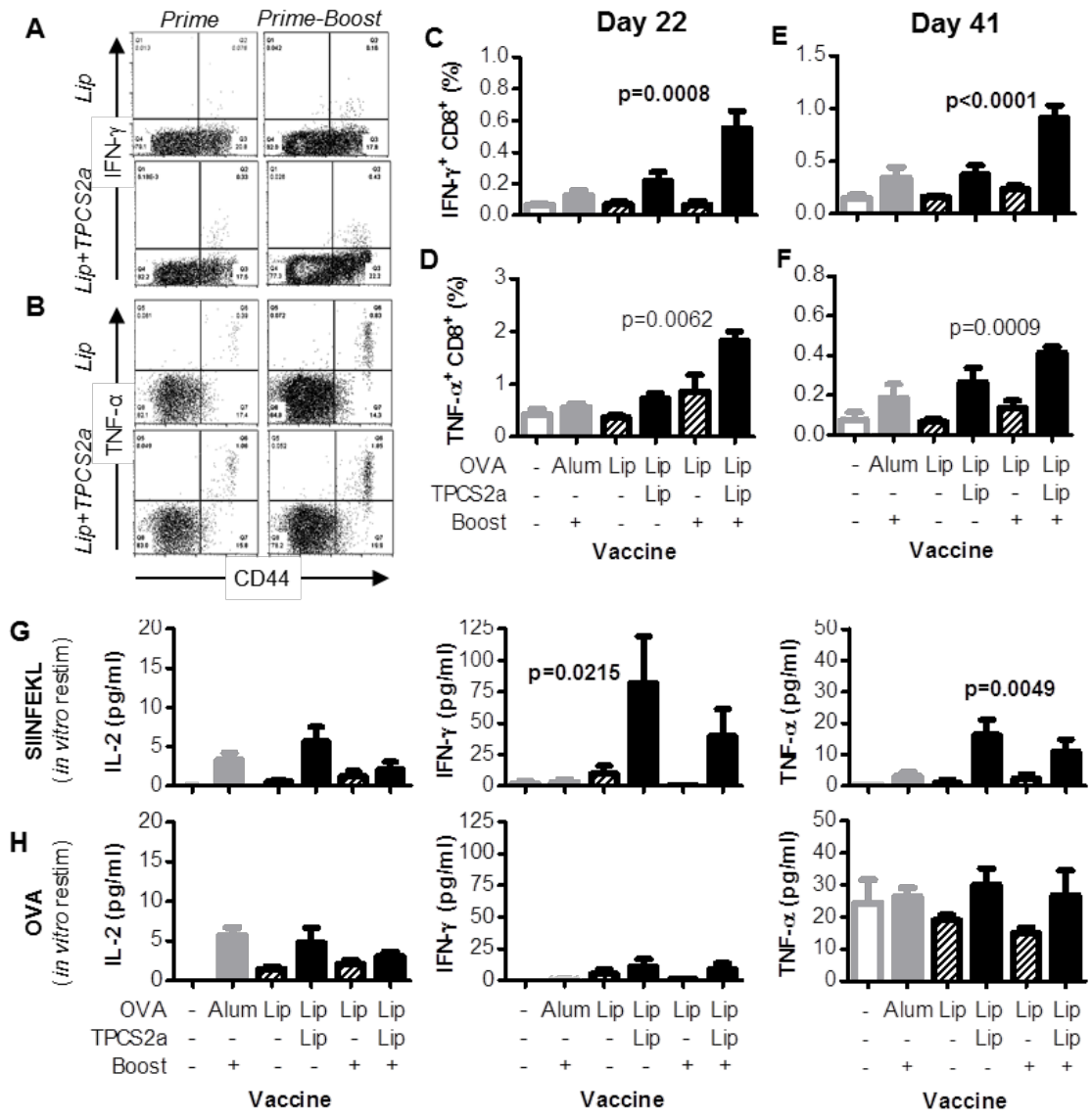

Figure 4: Cytokine secretion in lymphocytes from immunised mice. The mice primed and boosted as described in Fig. 3 were analysed for cytokine production by flow cytometry (**A-F**) and ELISA (**G-H**). Representative dot blots of intracellular IFN-γ (**A**) and TNF-α (**B**) producing and $CD44^+$ $CD8^+$ cells are shown. IFN-γ and TNF-α was analysed from blood (**A-D**; day 22) and from spleen cells (**E-F**; day 41). Spleen cells from day 41 were cultured *in vitro* with SIINFEKL peptide (**G**) or OVA protein (**H**), and the concentration of cytokines in the supernatants were analysed by ELISA. IL-2 was measured after 24 h and IFN-γ and TNF-α after 72 h. Histograms show means and SEM of cytokine producing cells. Hatched bars and filled black bars illustrate groups of mice that received liposomes with OVA without and with admixed TPCS2a-containign liposomes, resp. Untreated and Alum-OVA treated mice are illustrated with open and grey bars, resp. Statistical significance of the effect of TPCS2a treatment was analysed by 2-way ANOVA.

Antibody responses were reduced after immunisation with photosensitiser

Blood was collected and serum made for analysis of anti-OVA antibodies. A single immunisation with OVA-containing liposomes produced sero conversion of OVA-specific IgG1 (**Fig. 5A**) and IgG2c (**Fig. 5B**) as measured on day 41 after primary immunisation. The serum concentrations of both IgG subclasses were significantly reduced when mice were immunised with vaccines that also contained the photosensitiser TPCS2a ($p<0.01$ for IgG1 and 0.05 for IgG2c). When mice received a booster injection of liposomal OVA, a marked rise in anti-OVA IgG1 was observed. A second injection of liposomal OVA and TPCS2a also showed a strong IgG1 increase, with no statistical significant difference being observed between mice immunised twice with or without TPCS2a. The immunogenicity of two doses with liposomal OVA was also similar to that of two injections with aluminium-adsorbed OVA. The serum concentration of IgG2c, an antibody subclass more associated with stronger $CD4^+$ T-cell responses, also increased after booster injections, but here, a significant lower concentration was measured in mice immunised with TPCS2-containing liposomes than with TPCS2a-free liposomes ($p<0.05$).

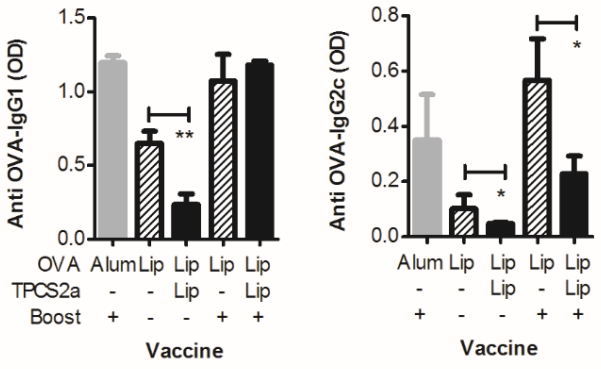

Figure 5: Antibody responses in immunised mice. The mice primed and boosted as described in Figure 3 were bled on day 41 for analysis of OVA-specific IgG1 (**A**) and IgG2c (**B**) antibodies in serum by ELISA. Histograms show means and SEM of optical density (OD) for 1:100 dilutions of individual sera. Hatched and filled black bars show mice that received liposomes without or with TPCS2a, respectively. Significant differences between groups treated with or without TPCS2a were analysed using Kruskal-Wallis: *: $p<0.05$; ** $p<0.01$.

Liposomes protected TPCS2a from light-induced inactivation

The use of liposomes expectedly improved uptake and antigen presentation *in vitro* with bone-marrow derived APCs and IC-21 cells lines (data not shown). To test if the liposomes protected the photosensitiser from light-induced inactivation, the change in fluorescent properties was measured over time under different light exposition. TPCS2a solutions and liposomal preparations of TPCS2a were exposed to daylight, and the natural daylight exposure was interrupted by dark cycles at night; control samples were kept light protected throughout the study. No difference in fluorescence was observed between liposomal or solute TPCS2a if kept protected from light (**Fig. 6A**). In the dark, TPCS2a fluorescence remained constant for 7 days for the preparations tested. When aqueous and liposomal

TPCS2a samples were exposed to daylight-night cycles, a stronger decay of TPCS2 fluorescence was observed for the aqueous photosensitiser solutions in PBS with 0.006% Tween 80. For the latter, approx. 15-20% fluorescence loss was measured within 1 day. The decay reached by day 7 a residual fluorescence of approx. 40% of the original fluorescence (**Fig. 6A**). For liposomal TPCS2a preparations, the fluorescence decay was less than 5% by day 1, the day 7 residual being approx. 80% of the original baseline fluorescence.

TPCS2a did not cause notable changes in the liposomes' physicochemical properties

As a cationic chemical, TPCS2a could also affect the physicochemical stability of the liposomes. Kept light-protected at 4 °C, particle size, polydispersity, and zeta potential altered only very slightly over 13 days storage (**Fig. 6B-D**). While embedded OVA had no measurable effect on liposome size stability over 13 days, TPCS2a caused a slight increase in the size from approx. 345 nm to 375 nm within one day, after which the size remained stable (**Fig. 6B**). The polydispersity index PDI remained similar for all three tested preparations, suggesting that size dispersity was not affected by TPCS2a or OVA encapsulation (**Fig. 6C**). Finally, the slightly negative zeta potential of the DPPC:Chol liposomes was not affected by TPCS2a or OVA incorporation, the baseline potentials being -6 to -5 mV for all three formulations. The cold and dark storage caused no particular change in the zeta potential of OVA-containing liposomes (**Fig. 6D**). Both empty and TPCS2a-containing liposomes showed an initial but small increase in the zeta potential, but by day 13, the potential lowered again to levels similar to the baseline levels, for which reason we conclude that TPCS2a did not compromise storage stability of the DPPC:Chol liposomes.

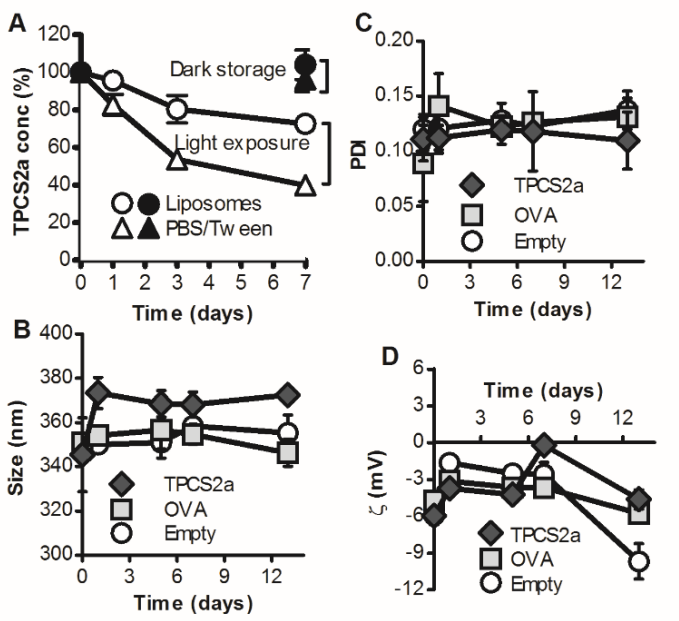

Figure 6: Stability of empty, TPCS2a-containing an OVA-containing liposomes *in vitro*. (**A**) Aqueous (triangles) or liposomal (circles) preparations of TPCS2a were stored in sealed 4 ml borosilicate glass vials under daylight exposure (open symbols) or under darkness (filled symbols). Samples were drawn at various time points and analysed for TPCS2a-specific fluorescence. (**B-D**) Empty, TPCS2a-containing, and OVA-containing liposomes were stored at 4 °C in the dark and analysed for size (**B**), size distribution (**C**), and zeta potential (**D**) as a function of time.

In agreement with size and zeta potential data, cryo-SEM micrographs did not reveal noteworthy differences in particle morphology between the empty and OVA- or TPCS2a-loaded liposomes nor between freshly prepared and stored liposomes (**Fig. 7**).

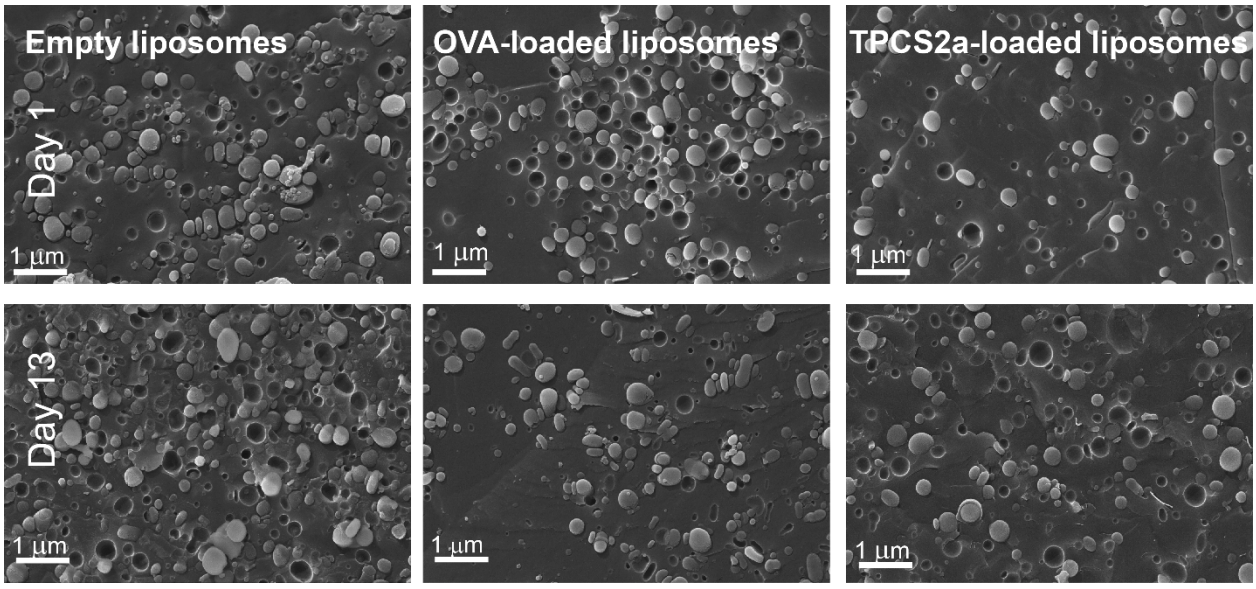

Figure 7: Morphology of empty, OVA-containing, and TPCS2a-containing liposomes as visualised by cryo-SEM. The liposomes were freshly prepared and frozen after 1 day for cryo-SEM or stored light-protected at 4 °C for 13 days before processing for cryo-SEM.

Discussion

To cope with this general problem that current vaccines only poorly lead to cross-presentation of MHC class I-restricted antigens and stimulation of cytotoxic $CD8^+$ T cells, we recently described a new vaccination method of immunisation where antigens are administered in combination with a photosensitiser [318-320]. After uptake of antigen and photosensitiser by phagocytosis or endocytosis into APCs, light exposure of the site of immunisation leads to disruption of photosensitised phagosomes and concomitant release of antigen into cytosol. This process enables antigen to access proteasomes for digestion with subsequent loading on MHC-I which precedes export of MHC-I-peptide complexes and $CD8^+$ T-cell priming (**Fig. 1**). The proof of principle of this so-called PCI-based immunisation method was ascertained using soluble antigen and photosensitiser. In the current study, we formulated the antigen OVA and the photosensitiser TPCS2a in DPPC liposomes in order to test if PCI-based immunisation for stimulation of $CD8^+$ T-cell responses could be achieved with vaccine particles.

The results clearly revealed that liposomes can be used for PCI-based cytosolic antigen targeting and $CD8^+$ T-cell cross priming. Antigen-specific $CD8^+$ T-cell proliferation improved strongly using TPCS2a-containing liposomes. This effect was observed after a single immunisation, but especially upon a second immunisation, which markedly boosted the long-lasting antigen-specific $CD8^+$ T-cell response, with high frequencies of antigen-specific $CD8^+$ T-cells observed in mice at least seven weeks after the primary immunisation. In contrast, secondary immunisation with TPCS2a-free liposomes did not notably boost $CD8^+$ T-cell responses. Control immunisation with antigen adsorbed on aluminium hydroxide showed that this adjuvant, at least in mice and with OVA, was able to mediate some cross presentation of antigen, comparable with TPCS2a-free liposomes, but the $CD8^+$ T-cell proliferation was clearly inferior to that of PCI-based immunisation with liposomes.

Similar effects of PCI-based immunisation were observed with regards to the production and secretion of the effector cytokines IFN-γ and TNF-α. Both cytokines were up-regulated after immunisation with OVA- and TPCS2a-containing liposomes, and the effect was strictly dependent on the photosensitiser. In contrast, the production of cytokines after immunisation with aluminium-based vaccine and with TPCS2a-free liposomes was more ambiguous. IFN-γ and TNF-α, but not IL-2 secretion was markedly lower than after immunisation with the photosensitive vaccines. After restimulation of splenocytes with antigen *in vitro*, both aluminium-based and liposome-based vaccines resulted in high levels of IL-2 secretion similar to that of PCI-based vaccine. This result fits with the proliferation data as much as IL-2 is a growth factor for both $CD4^+$ and $CD8^+$ T-cells and especially needed in the primary expansion

phase of the cells [321, 322]. Indeed, high levels of IL-2 were also observed in splenocytes restimulated *in vitro* with the full OVA protein, able to stimulate T cells of several subsets and specificities. The relatively low levels in IL-2 after restimulation of splenocytes from mice immunised with PCI-based vaccines with SIINFEKL, might be associated with a faster consumption of IL-2 by the more reactive and faster proliferating cells from PCI-vaccinated mice.

While restimulation of splenocytes with the CD8 epitope SIINFEKL showed a clear positive effect of PCI-based vaccination in terms of SIINFEKL-specific secretion of IFN-γ and TNF-α, restimulation with OVA protein did not significantly distinguish PCI-based vaccines from non-PCI vaccines, nor liposomal vaccines from alum-based vaccines. This is most likely due the MHC-II-mediated $CD4^+$ T-cell responses stimulated by non-PCI vaccines, but not by PCI-based vaccines, which are thought to act by diverting the antigen away from MHC-II processing when the photosensitised phagosomes are photoactivated and release their content of antigen into cytosol for MHC-I processing and presentation (**Fig. 1**). By consequence, this would lead to reduced MHC-II-mediated immune responses in vaccinees receiving the PCI-based vaccines. Indeed, MHC-II-dependent antibody responses in mice given PCI-based vaccines were significantly reduced as compared to mice that received non-PCI liposomal vaccines or alum-based vaccines. A single injection of TPCS2a-containing vaccines triggered little IgG1 and IgG2c production, while both subclasses were significantly elevated upon a second immunisation session two weeks after the first session, suggesting that not all antigen escape MHC class II antigen presentation in PCI-based vaccination. This mixed MHC-I and MHC-II presentation might result from antigen and photosensitiser being embedded in separate liposomes that may target different antigen presenting cells. Only when both antigen and photosensitiser are targeted to the same individual APC, the PCI-mediated triggering of MHC-I-restricted $CD8^+$ responses is expected. Future studies will need to address this question, but technological and analytical issues related to the mixing of the lipids with the disulphonated photosensitiser TPCS2a and with a large protein antigen need to be solved first.

As shown in numerous studies in the last decades, liposomes can target antigens and drugs to phagocytic cells such as professional APCs thanks to their particulate nature as well as membrane-fusogenic properties. Such APC-targeting properties were also aimed for in the current study with photoactive liposomal vaccines, but liposomes may potentially also protect photosensitisers from light-induced inactivation. This latter would simplify production, storage, and handling of such preparations, and it would even prevent premature activation *in vivo* due to ambient environmental light. Indeed, for soluble TPCS2a stored at day light, more than 60% of the compound was lost within 24 hours as measured by fluorescence. In comparison, liposomal preparations of TPCS2a lost less than 5% of the fluorescence within 24 hours and only 20% over 7 days. Hence, although it may still be

important to assure light protection during storage of TPCS2a-based vaccines, the observations made here may suggest a safety benefit of liposomal PCI-based vaccines over soluble PCI-based vaccines.

Conclusion

The current study demonstrated that PCI-based immunisation technology can be applied to particulate vaccines such as liposomes. DPPC liposomes containing the photosensitiser TPCS2a significantly improved cross-priming of $CD8^+$ T cells. In addition, the study contributed to the understanding of the mechanism of PCI-based immunisation showing a clear diversion of antigen from MHC-II to MHC-I processing. Hence, PCI-based vaccines have the potential to add to current vaccine practice a new method of vaccination that, as opposed to current vaccines, can stimulate strong $CD8^+$ T-cell responses. We expect this method of vaccination to enable treatment and prophylaxis of $CD8^+$ T-cell-sensitive infections and cancers. Since the photosensitiser TPCS2a, in combination with a cytostatic drug, is already approved for clinical trials in humans with cancer (clinicaltrial.gov: NCT01872923, NCT00993512, NCT01900158, NCT01606566), the translation of this vaccination modality into clinical setting should be facilitated.

Chapter 6

General discussion and perspectives

General discussion and perspectives

The presented PhD thesis describes the development and the immunological testing of various liposomal vaccines for their applicability and capacity to target antigens and adjuvants to specific immune cells and cellular compartments. In vaccination and immunotherapy, both T cells and B cells play important but distinct roles in forming and providing protective immune responses. Current prophylactic vaccines but also therapeutic allergy vaccines, i.e., AIT, require the coordinated interaction of APCs, T cells and B cells, and primarily provide protection by eliciting pathogen-, toxin-, or allergen-specific antibodies [121-123]. However, due to involvement of these innate and adaptive immune cells, the time needed for B-cell activation and secretion of protective serum concentrations of antigen-specific, isotype-switched, and high-affinity antibodies is long. In contrast, TI antigens can directly stimulate B cells for secretion of antibodies, a reaction that can take place within few days. Hence, a vaccine that gives rapid rise to protective levels of antibodies without involvement of T cells can be beneficial. Such vaccine strategies have not been systematically explored yet due to lack of evidence on TI isotype switch with protein antigens and protective B-cell memory.

In the last decade, our understanding of TI antigens has grown substantially [8, 61, 62, 228]. Most studies on TI immune responses have been performed with polysaccharide antigens or haptenated polysaccharides, of no direct clinical relevance. In *chapter 2*, I describe own studies where peptides stimulated class switch and, for the first time, *in vivo* B-cell memory recall responses in the absence of T cells. Such responses were achieved by formulating liposomes with a dense assembly of peptide and monophosphoryl lipid A (MPLA) on the surface [202, 228]. The liposomal vaccine directly stimulated B cells by a novel immune mechanism with characteristics of both type 1 (TI-1) and type 2 (TI-2) antibody responses. Furthermore, the antibody secretion and class switch took place within 2-4 days of immunization, and it was associated with formation of long-lived GCs and antibody affinity maturation. Such liposomal TI vaccines could serve as a valuable tool for further investigation of TI immune responses and a step closer to the realization of TI vaccination.

Although approximately one third of the world population suffers from allergy due to sensitization to environmental proteins, and despite the documented long-term curative effect of AIT [144], AIT is taken up by less than 10% of the patients. The poor uptake and treatment compliance are mainly due to the risk of treatment-associated side effects and long duration of AIT. Hence, current research focuses on increasing safety and enhancing efficacy of AIT. It is well documented that AIT induces both B- and T-cell responses, with regulatory CD4 T cells playing an important role in immune tolerance and the major effect of IgG antibodies being the neutralization of allergen. However, the relative

importance of B- and T-cell responses in AIT is frequently subject to discussions and research [260, 264, 265]. The facts that transferred antiserum can provide protection against allergic reaction in the host [259, 260], and that protection against anaphylaxis must take place within minutes of allergen exposure, indicates an important effector role of circulating allergen-specific antibodies, although the induction of such antibodies during AIT may still be regulated by T cells. The goal of *chapter 3* was to use the TI liposomal vaccine described in *chapter 2* to address the question if efficacy of AIT was not only affected by, but also dependent on the contribution of T cells. The ovalbumin protein adsorbed on the adjuvant aluminum hydroxide (alum) was used as TD control vaccine in AIT. The liposomal TI vaccine induced high IgG titers in untreated mice by direct stimulation of B cells in *chapter 2*, yet when used in AIT in previously sensitized mice in *chapter 3*, IgG1 and IgG2a levels in blood did not further increase. Additionally, the AIT with the liposomal TI vaccine did not provide relief from anaphylactic reactions upon allergen challenge. However, in *chapter 2*, the TI antibodies were found to have neutralizing effects in prophylactic models of allergy and infection. Further studies are needed to determine whether this lack of efficacy in AIT is due to the general inability of TI antigens to further increase IgG levels in blood or due to T-cell requirement in AIT.

The results of experiments performed in *chapter 3* suggest better AIT efficacy when longer dosing intervals between each AIT session were applied. This result is also in line with the conclusion made in *chapter 4* where higher IgG2a levels, more antibody affinity maturation, and improved protection against allergic anaphylaxis was observed when AIT intervals increased in intralymphatic immunotherapy (ILIT) in mice. These results are in agreement with clinical observations, showing that ILIT was found to reduce effectively allergic symptoms when administered with four-week intervals [273-275], but not with two-week intervals [276]. Of note, the general recommendations for administration of vaccines include injection intervals of at least four weeks, and vaccination is considered invalid if the injection interval is shortened to less than the recommended minimum [268-271]. As persistent high doses of antigen may have an inhibitory effect on the activation of high-affinity B cells, longer vaccination intervals in AIT maintain tolerance of those cells [272]. The daily use of SLIT is the current practice and may oppose the concept of long intervals [133]. However, the fact that more frequent and higher doses are administered in SLIT than SCIT [133], indicates importance of the administration route. Sentinel DCs in the oral mucosa are likely rather prone to induce tolerance than to be immune stimulatory. Therefore, the contradiction in SLIT dosing might be explained by the evolutionarily conserved mechanism of oral tolerance, which ensures immune tolerance to environmental stimuli such as food and commensal bacteria [323]. The research presented in this thesis was conducted in mice, for which reason the results cannot be directly translated to humans

without further investigation. However, dosing intervals in ILIT affected the immunogenicity and treatment efficacy in mice and should be considered in clinical research and practice.

One of the greatest challenges in vaccinology is the stimulation of cytotoxic CD8 T cell (CTL) responses. For this, the antigen must reach the cytosol of APCs, be processed by the proteasome, and loaded on MHC class I molecules for presentation to CD8 T cells [182]. As a rule of thumb, vaccine peptide and protein antigens are typically processed in phagolysosomes and loaded on MHC class II molecules for presentation to CD4 T cells [184]. Recently, our group demonstrated that targeting soluble antigens to the cytosol can be achieved with photosensitizers and light, so-called photochemical internalization (PCI) [320]. In *chapter 5*, the cytosolic delivery is further improved by utilizing liposomes to carry the photosensitizer and the ovalbumin protein to APCs, resulting in strongly improved antigen-specific CD8 T-cell proliferation and effector functions. By consequence, the MHC II-mediated immune responses, especially antibody response, were considerably reduced. Furthermore, entrapment in liposomes protected the photosensitizer from light-induced inactivation upon storage, thereby increasing shelf time. Hence, photochemical internalization in combination with liposomes may serve as an effective adjuvant in vaccination where strong CD8 T-cell responses are needed, e.g. in cancer and upon infection with intracellular pathogens.

The main limitations of the research presented in this PhD thesis include the demonstration of memory responses in *chapter* 2. Currently, the memory is being measured only as a boost in serum antibodies upon secondary immunization and the memory B-cell population not being directly identified. Further characterization of the phenotypical and functional features of the population of memory B cells, such as the B-cell subtype involved, expressed surface markers etc., would provide information on how memory B cells induced with TI antigens differ from those induced with TD antigens. Such knowledge would be important for vaccine development and continuing research. Similarly, it remains to be determined which signals prevent the GCs from dissipating in the absence of positive selection by T cells. In addition, determining the B-cell subsets involved in such GCs as well as whether activation-induced cytidine deaminase (AID) is expressed, and if affinity maturation takes place in the GCs or outside the GCs would be valuable in expanding our general understanding of TI immune responses. In *chapter 3*, avidity of the antibodies induced by different treatments was not compared, although the blocking activity of IgG antibodies in AIT may depend on their affinity for the allergen rather than quantity of antibodies [132, 141]. As AIT with the liposomal TI vaccine surprisingly did not increase antibody levels more than already established during sensitization of mice, it would be interesting to see if the avidity of the antibodies improved. Finally, the photosensitizer and ovalbumin could not be formulated together in the same liposomal preparation for the studies in *chapter 5*. Hence, as the

concept of photochemical internalization would assume that antigen and photosensitizer reach the same APCs, in the current studies, the APCs must simultaneously phagocytose both types of liposomes for successful delivery to the cytosol and MHC class I antigen presentation. Nonetheless, concurrent uptake of the two types of liposomes appeared to take place as immunization resulted in improved CD8 T-cell activation. Perhaps delivery could be enhanced even further with co-delivery of the photosensitizer with antigen. Recent work in our group has shown that co-encapsulation of photosensitizer and ovalbumin in liposomes is feasible, and immunological studies on the new preparations are underway.

In conclusion, liposomes can be utilized to target peptide antigen to B cells and to activate these without any T-cell involvement. In vaccination, patients suffering from T-cell deficiencies such as HIV, or T-cell dependent B-cell immunodeficiencies such as common variable immune deficiency (CVID), or patients treated with immune suppressive drugs, or the elderly with aged and inefficient T-cell populations could benefit from TI vaccines [255]. Moreover, in vaccination against non-tumor self-antigens such as amyloid β in Alzheimer's disease, it is important to avoid elicitation of cytotoxic T cells that may cause unwanted autoimmune tissue damage [256]. Also here, TI vaccines may serve a valuable purpose. TI vaccines may prove useful in diseases where rapid immune protection and immunization coverage is needed, but where conventional TD vaccines are not recommended, justified, or not fast or efficient enough, e.g. for the protection of populations against epidemic pathogens during infectious outbreaks or against bioterrorism with infectious agents [8, 117, 257].

The utilization of TI vaccine strategies have not been extensively explored, since TI protein antigens have been considered unable to stimulate generation of long-lasting IgG antibodies as well as B-cell memory [61]. In light of the growing body of evidence on protective B-cell memory forming without T-cell help, there is a reason to revisit the true potential of TI vaccines [8, 61, 62, 228]. In this context, liposomes represent an attractive option for TI vaccine development as adjuvants and peptide antigens can be attached on the liposomal surface with densities sufficient to bypass the need for T-cell help [202, 228]. Furthermore, formulating photosensitizer in liposomes has the potential to add new options to current vaccine practice by stimulating strong CD8 T-cell responses, for instance in cancer immunotherapy or in therapeutic vaccination to fight chronic intracellular pathogens.

Appendices

Curriculum vitae

Acknowledgements

List of abbreviations

References

Curriculum vitae

Name: HJÁLMSDÓTTIR
First name: Ásdís
Date of birth: October 28 1985
Nationality: Icelandic

Education

Aug 2001 – May 2005	Secondary school, Menntaskólinn við Hamrahlíð Graduated from the line of Natural Sciences
Aug 2005 – Jun 2009	B.Sc. in Pharmacy, University of Iceland
Sep 2009 – Feb 2012	M.Sc. in Pharmacy, University of Iceland Master's thesis titled "The effects of Misoprostol on uterine activity in rats"
Nov 2013 – Today	PhD in Immunology, University of Zurich

Publications

Hjálmsdóttir et al. *Dosing intervals in intralymphatic immunotherapy*. Clinical & Experimental Allergy, 2016. 46(3): 504-507.

Hjálmsdóttir et al. *Cytosolic delivery of liposomal vaccines by means of the concomitant photosensitization of phagosomes*. Molecular pharmaceutics, 2016. 13(2): 320-329.

Hjálmsdóttir et al. *A novel type of peptide-induced T-cell independent B-cell responses with class switch and memory using liposomes*. Submitted for publication, 2018.

Other activities

I have represented Iceland in all Major Championships in athletics since 2008, qualifying for the women's javelin throw final at the 2010 and 2016 European Championships, the 2017 World Championships, and the 2012 Olympic Games.

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I embarked on my PhD journey back in November 2013 when I decided to dive right into the deep end of the pool. As a pharmacist, I had very limited background knowledge of immunology and yet Pål gave me the opportunity to take on this exciting project. I quickly realized that this would be quite the challenge. However, I have never been known to shy away from a good challenge and with the support and contribution of my colleagues and my family, this thesis saw the light of day.

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List of abbreviations

MZ	Marginal zone
TD	T-cell dependent
TI	T-cell independent
BCR	B-cell receptor
APC	Antigen presenting cell
FDC	Follicular dendritic cell
DC	Dendritic cell
MHC	Major histocompatibility complex
T_H	T-helper cell
Ig	Immunoglobulin
IL	Interleukin
IFN	Interferon
PC	Plasma cell
GC	Germinal center
BTK	Bruton‘s tyrosine kinase
TLR	Toll-like receptor
LPS	Lipopolysaccharide
SHM	Somatic hypermutation
CSR	Class switch recombination
T_{FH}	Follicular helper T cell
QM	Quasimonoclonal
BM	Bone marrow
FcR	Fc Receptor
TGF	Tumor growth factor
AID	Activation-induced cytidine deaminase
ADCC	Antibody-dependent cellular cytotoxicity
NK	Natural killer
PAMP	Pathogen-associated molecular patterns
PRR	Pattern recognition receptor
VLP	Virus-like particle
AIT	Allergen immunotherapy
SCIT	Subcutaneous immunotherapy
SLIT	Sublingual immunotherapy
OIT	Oral immunotherapy
ILIT	Intralymphatic Immunotherapy
T_{reg}	Regulatory T cell
CTL	Cytotoxic $CD8^+$ T cells
MPLA	Monophosphoryl lipid A
DAMP	Danger-associated molecular patterns
OVA	Ovalbumin
PCI	Photochemical internalization
TPCS2a	Tetraphenyl chlorine disulfonate
DPPC	Dipalmitoyl phosphatidylcholine
Chol	Cholesterol
CVID	Common varible immune deficiency

References

1. Parker, D.C., *T cell-dependent B cell activation.* Annual review of Immunology, 1993. **11**(1): p. 331-360.
2. Martin, F. and J.F. Kearney, *B1 cells: similarities and differences with other B cell subsets.* Current Opinion in Immunology, 2001. **13**(2): p. 195-201.
3. Allman, D. and S. Pillai, *Peripheral B cell subsets.* Current Opinion in Immunology, 2008. **20**(2): p. 149-157.
4. Martin, F., A.M. Oliver, and J.F. Kearney, *Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens.* Immunity, 2001. **14**(5): p. 617-629.
5. Liu, Y.J., S. Oldfield, and I. Maclennan, *Memory B cells in T cell-dependent antibody responses colonize the splenic marginal zones.* European Journal of Immunology, 1988. **18**(3): p. 355-362.
6. Zandvoort, A. and W. Timens, *The dual function of the splenic marginal zone: Essential for initiation of anti-TI-2 responses but also vital in the general first-line defense against blood-borne antigens.* Clinical & Experimental Immunology, 2002. **130**(1): p. 4-11.
7. Guinamard, R., et al., *Absence of marginal zone B cells in Pyk-2-deficient mice defines their role in the humoral response.* Nature immunology, 2000. **1**(1): p. 31-36.
8. Defrance, T., M. Taillardet, and L. Genestier, *T cell-independent B cell memory.* Current Opinion in Immunology, 2011. **23**(3): p. 330-336.
9. Vinuesa, C.G. and P.-P. Chang, *Innate B cell helpers reveal novel types of antibody responses.* Nature immunology, 2013. **14**(2): p. 119-126.
10. Alugupalli, K.R., et al., *B1b lymphocytes confer T cell-independent long-lasting immunity.* Immunity, 2004. **21**(3): p. 379-390.
11. Oliver, A.M., F. Martin, and J.F. Kearney, *IgMhighCD21high lymphocytes enriched in the splenic marginal zone generate effector cells more rapidly than the bulk of follicular B cells.* Journal of Immunology, 1999. **162**(12): p. 7198-7207.
12. Senti, G., P. Johansen, and T.M. Kündig, *Intralymphatic immunotherapy.* Current Opinion in Allergy and Clinical Immunology, 2009. **9**(6): p. 537-543.
13. Kaldjian, E.P., et al., *Spatial and molecular organization of lymph node T cell cortex: a labyrinthine cavity bounded by an epithelium-like monolayer of fibroblastic reticular cells anchored to basement membrane-like extracellular matrix.* International Immunology, 2001. **13**(10): p. 1243-1253.
14. Sainte-Marie, G., F.S. Peng, and C. Belisle, *Overall architecture and pattern of lymph flow in the rat lymph node.* Developmental Dynamics, 1982. **164**(4): p. 275-309.
15. Willard-Mack, C.L., *Normal structure, function, and histology of lymph nodes.* Toxicologic Pathology, 2006. **34**(5): p. 409-424.
16. Metlay, J.P., E. Puré, and R.M. Steinman, *Control of the immune response at the level of antigen-presenting cells: a comparison of the function of dendritic cells and B lymphocytes.* Advances in Immunology, 1989. **47**: p. 45-116.
17. Cyster, J.G., *B cell follicles and antigen encounters of the third kind.* Nature immunology, 2010. **11**(11): p. 989-996.
18. Stoll, S., et al., *Dynamic imaging of T cell-dendritic cell interactions in lymph nodes.* Science, 2002. **296**(5574): p. 1873-1876.
19. Crotty, S., *Follicular helper CD4 T cells (Tfh).* Annual review of Immunology, 2011. **29**: p. 621-663.
20. Bretscher, P. and M. Cohn, *A theory of self-nonself discrimination.* Science, 1970. **169**(3950): p. 1042-1049.
21. Gershon, R.K. and K. Kondo, *Cell interactions in the induction of tolerance: the role of thymic lymphocytes.* Immunology, 1970. **18**(5): p. 723.
22. Noelle, R.J. and E.C. Snow, *Cognate interactions between helper T cells and B cells.* Immunology Today, 1990. **11**(10): p. 361-368.

23. Kuchen, S., et al., *Essential role of IL-21 in B cell activation, expansion, and plasma cell generation during CD4+ T cell-B cell collaboration.* Journal of Immunology, 2007. **179**(9): p. 5886-5896.
24. Cannons, J.L., et al., *Optimal germinal center responses require a multistage T cell: B cell adhesion process involving integrins, SLAM-associated protein, and CD84.* Immunity, 2010. **32**(2): p. 253-265.
25. Vinuesa, C.G., et al., *Follicular B helper T cells in antibody responses and autoimmunity.* Nature Reviews Immunology, 2005. **5**(11): p. 853-865.
26. Smith, K.G., et al., *The phenotype and fate of the antibody-forming cells of the splenic foci.* European Journal of Immunology, 1996. **26**(2): p. 444-448.
27. MacLennan, I., et al., *Extrafollicular antibody responses.* Immunological reviews, 2003. **194**(1): p. 8-18.
28. Mond, J.J., A. Lees, and C.M. Snapper, *T cell-independent antigens type 2.* Annual review of Immunology, 1995. **13**(1): p. 655-692.
29. Mosier, D., J. Mond, and E. Goldings, *The ontogeny of thymic independent antibody responses in vitro in normal mice and mice with an X-linked B cell defect.* Journal of Immunology, 1977. **119**(6): p. 1874-1878.
30. Bekeredjian-Ding, I. and G. Jego, *Toll-like receptors–sentries in the B-cell response.* Immunology, 2009. **128**(3): p. 311-323.
31. de Vinuesa, C.G., et al., *T-independent type 2 antigens induce B cell proliferation in multiple splenic sites, but exponential growth is confined to extrafollicular foci.* European Journal of Immunology, 1999. **29**(4): p. 1314-1323.
32. Klein, U. and R. Dalla-Favera, *Germinal centres: role in B-cell physiology and malignancy.* Nature Reviews Immunology, 2008. **8**(1): p. 22-33.
33. Berek, C., A. Berger, and M. Apel, *Maturation of the immune response in germinal centers.* Cell, 1991. **67**(6): p. 1121-1129.
34. Di Noia, J.M. and M.S. Neuberger, *Molecular mechanisms of antibody somatic hypermutation.* Annual Review of Biochemistry, 2007. **76**: p. 1-22.
35. Victora, G.D. and M.C. Nussenzweig, *Germinal centers.* Annual review of Immunology, 2012. **30**: p. 429-457.
36. Jacob, J., R. Kassir, and G. Kelsoe, *In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl) acetyl. I. The architecture and dynamics of responding cell populations.* Journal of Experimental Medicine, 1991. **173**(5): p. 1165-1175.
37. Kroese, F., W. Timens, and P. Nieuwenhuis, *Germinal center reaction and B lymphocytes: morphology and function*, in *Reaction Patterns of the lymph node. Current Topics in Pathology*, E. Grundmann and E. Vollmer, Editors. Vol. 1990, Springer: Berlin, Heidelberg. p. 103-148.
38. MacLennan, I.C., *Germinal centers.* Annual review of Immunology, 1994. **12**(1): p. 117-139.
39. Basso, K., et al., *Integrated biochemical and computational approach identifies BCL6 direct target genes controlling multiple pathways in normal germinal center B cells.* Blood, 2010. **115**(5): p. 975-984.
40. Allen, C.D., T. Okada, and J.G. Cyster, *Germinal-center organization and cellular dynamics.* Immunity, 2007. **27**(2): p. 190-202.
41. Reinhardt, R.L., H.-E. Liang, and R.M. Locksley, *Cytokine-secreting follicular T cells shape the antibody repertoire.* Nature immunology, 2009. **10**(4): p. 385-393.
42. Shapiro-Shelef, M. and K. Calame, *Regulation of plasma-cell development.* Nature Reviews Immunology, 2005. **5**(3): p. 230-242.
43. Pape, K.A., et al., *Different B cell populations mediate early and late memory during an endogenous immune response.* Science, 2011. **331**(6021): p. 1203-1207.
44. Gray, D., *Immunological memory.* Annual review of Immunology, 1993. **11**(1): p. 49-77.
45. Suan, D., C. Sundling, and R. Brink, *Plasma cell and memory B cell differentiation from the germinal center.* Current Opinion in Immunology, 2017. **45**: p. 97-102.
46. Elgueta, R., et al., *CCR6-dependent positioning of memory B cells is essential for their ability to mount a recall response to antigen.* Journal of Immunology, 2015. **194**(2): p. 505-513.

47. Dufaud, C.R., L.J. McHeyzer-Williams, and M.G. McHeyzer-Williams, *Deconstructing the germinal center, one cell at a time.* Current Opinion in Immunology, 2017. **45**: p. 112-118.
48. Schwickert, T.A., et al., *In vivo imaging of germinal centres reveals a dynamic open structure.* Nature, 2007. **446**(7131): p. 83-87.
49. Allen, C.D., et al., *Imaging of germinal center selection events during affinity maturation.* Science, 2007. **315**(5811): p. 528-531.
50. Hauser, A.E., et al., *Definition of germinal-center B cell migration in vivo reveals predominant intrazonal circulation patterns.* Immunity, 2007. **26**(5): p. 655-667.
51. Lentz, V.M. and T. Manser, *Cutting edge: germinal centers can be induced in the absence of T cells.* Journal of Immunology, 2001. **167**(1): p. 15-20.
52. de Vinuesa, C.G., et al., *Germinal Centers without T Cells.* Journal of Experimental Medicine, 2000. **191**(3): p. 485-494.
53. Toellner, K.-M., et al., *Low-level hypermutation in T cell–independent germinal centers compared with high mutation rates associated with T cell–dependent germinal centers.* Journal of Experimental Medicine, 2002. **195**(3): p. 383-389.
54. Sze, D.M.-Y., et al., *Intrinsic constraint on plasmablast growth and extrinsic limits of plasma cell survival.* Journal of Experimental Medicine, 2000. **192**(6): p. 813-822.
55. Kepler, T.B. and A.S. Perelson, *Cyclic re-entry of germinal center B cells and the efficiency of affinity maturation.* Immunology today, 1993. **14**(8): p. 412-415.
56. Hosokawa, T., et al., *Studies on B-cell memory. III. T-dependent aspect of B memory generation in mice immunized with T-independent type-2 (TI-2) antigen.* Immunology, 1984. **53**(1): p. 97-104.
57. Le Moal, M.A. and P. Truffa-Bachi, *Immune memory expression to Tnp-Ficoll in CB. 20 mice: evidence for a multigenic control.* Cellular immunology, 1985. **95**(2): p. 428-436.
58. Zhang, J., et al., *B cell memory to thymus-independent antigens type 1 and type 2: the role of lipopolysaccharide in B memory induction.* European Journal of Immunology, 1988. **18**(9): p. 1417-1424.
59. Brodeur, P.H. and H.H. Wortis, *Regulation of thymus-independent responses: unresponsiveness to a second challenge of TNP-Ficoll is mediated by hapten-specific antibodies.* Journal of Immunology, 1980. **125**(4): p. 1499-1505.
60. Hosokawa, T., *Studies on B-cell memory. II. T-cell independent antigen can induce B-cell memory.* Immunology, 1979. **38**(2): p. 291-299.
61. Obukhanych, T.V. and M.C. Nussenzweig, *T-independent type II immune responses generate memory B cells.* Journal of Experimental Medicine, 2006. **203**(2): p. 305-310.
62. Taillardet, M., et al., *The thymus-independent immunity conferred by a pneumococcal polysaccharide is mediated by long-lived plasma cells.* Blood, 2009. **114**(20): p. 4432-4440.
63. Lesinski, G.B. and M.J. Westerink, *Novel vaccine strategies to T-independent antigens.* Journal of Microbiological Methods, 2001. **47**(2): p. 135-149.
64. O'Brien, K.L., M. Hochman, and D. Goldblatt, *Combined schedules of pneumococcal conjugate and polysaccharide vaccines: is hyporesponsiveness an issue?* The Lancet Infectious Diseases, 2007. **7**(9): p. 597-606.
65. Slifka, M.K., et al., *Humoral immunity due to long-lived plasma cells.* Immunity, 1998. **8**(3): p. 363-372.
66. Manz, R.A., A. Thiel, and A. Radbruch, *Lifetime of plasma cells in the bone marrow.* Nature, 1997. **388**(6638): p. 133-134.
67. Manz, R.A., et al., *Maintenance of serum antibody levels.* Annual Review of Immunology, 2005. **23**: p. 367-386.
68. Williams, A.F. and A.N. Barclay, *The immunoglobulin superfamily—domains for cell surface recognition.* Annual review of Immunology, 1988. **6**(1): p. 381-405.
69. Schroeder, H.W. and L. Cavacini, *Structure and function of immunoglobulins.* Journal of Allergy and Clinical Immunology, 2010. **125**(2): p. S41-S52.
70. Padlan, E.A., *Anatomy of the antibody molecule.* Molecular Immunology, 1994. **31**(3): p. 169-217.

71. Stavnezer, J., *Immunoglobulin class switching.* Current Opinion in Immunology, 1996. **8**(2): p. 199-205.
72. Vidarsson, G., G. Dekkers, and T. Rispens, *IgG subclasses and allotypes: from structure to effector functions.* Frontiers in immunology, 2014. **5**: p. 17.
73. Honjo, T., *Immunoglobulin genes.* Annual review of Immunology, 1983. **1**(1): p. 499-528.
74. Rothman, P., et al., *Structure and expression of germ line immunoglobulin heavy-chain epsilon transcripts: interleukin-4 plus lipopolysaccharide-directed switching to C epsilon.* Molecular and Cellular Biology, 1990. **10**(4): p. 1672-1679.
75. Berton, M.T., J.W. Uhr, and E.S. Vitetta, *Synthesis of germ-line gamma 1 immunoglobulin heavy-chain transcripts in resting B cells: induction by interleukin 4 and inhibition by interferon gamma.* Proceedings of the National Academy of Sciences, 1989. **86**(8): p. 2829-2833.
76. Shockett, P. and J. Stavnezer, *Effect of cytokines on switching to IgA and alpha germline transcripts in the B lymphoma I. 29 mu. Transforming growth factor-beta activates transcription of the unrearranged C alpha gene.* Journal of Immunology, 1991. **147**(12): p. 4374-4383.
77. McIntyre, T.M., et al., *Transforming growth factor beta 1 selectivity stimulates immunoglobulin G2b secretion by lipopolysaccharide-activated murine B cells.* Journal of Experimental Medicine, 1993. **177**(4): p. 1031-1037.
78. Snapper, C.M. and W.E. Paul, *Interferon-and B cell stimulatory factor-1 reciprocally regulate Ig isotype production.* Science, 1987. **236**(4804): p. 944-947.
79. Severinson, E., C. Fernandez, and J. Stavnezer, *Induction of germ-line immunoglobulin heavy chain transcripts by mitogens and interleukins prior to switch recombination.* European Journal of Immunology, 1990. **20**(5): p. 1079-1084.
80. Zelazowski, P., et al., *Antigen receptor cross-linking differentially regulates germ-line CH ribonucleic acid expression in murine B cells.* Journal of Immunology, 1995. **154**(3): p. 1223-1231.
81. Warren, W.D. and M.T. Berton, *Induction of germ-line gamma 1 and epsilon Ig gene expression in murine B cells. IL-4 and the CD40 ligand-CD40 interaction provide distinct but synergistic signals.* Journal of Immunology, 1995. **155**(12): p. 5637-5646.
82. Muramatsu, M., et al., *Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme.* Cell, 2000. **102**(5): p. 553-563.
83. Boes, M., *Role of natural and immune IgM antibodies in immune responses.* Molecular Immunology, 2000. **37**(18): p. 1141-1149.
84. Geisberger, R., M. Lamers, and G. Achatz, *The riddle of the dual expression of IgM and IgD.* Immunology, 2006. **118**(4): p. 429-437.
85. Martin, R.M., A. Silva, and A.M. Lew, *The Igh-1 sequence of the non-obese diabetic (NOD) mouse assigns it to the IgG2c isotype.* Immunogenetics, 1997. **46**(2): p. 167-168.
86. Martin, R.M., J.L. Brady, and A.M. Lew, *The need for IgG2c specific antiserum when isotyping antibodies from C57BL/6 and NOD mice.* Journal of Immunological Methods, 1998. **212**(2): p. 187-192.
87. Collins, A.M., *IgG subclass co-expression brings harmony to the quartet model of murine IgG function.* Immunology and Cell Biology, 2016. **94**(10): p. 949-954.
88. Sarma, J.V. and P.A. Ward, *The complement system.* Cell and tissue research, 2011. **343**(1): p. 227-235.
89. Bruhns, P. and F. Jönsson, *Mouse and human FcR effector functions.* Immunological reviews, 2015. **268**(1): p. 25-51.
90. Neuberger, M.S. and K. Rajewsky, *Activation of mouse complement by monoclonal mouse antibodies.* European Journal of Immunology, 1981. **11**(12): p. 1012-1016.
91. Finkelman, F., et al., *IFN-gamma regulates the isotypes of Ig secreted during in vivo humoral immune responses.* Journal of Immunology, 1988. **140**(4): p. 1022-1027.
92. Nimmerjahn, F. and J.V. Ravetch, *Divergent immunoglobulin g subclass activity through selective Fc receptor binding.* Science, 2005. **310**(5753): p. 1510-1512.

93. Jegaskanda, S., et al., *Antibody-dependent cellular cytotoxicity is associated with control of pandemic H1N1 influenza virus infection of macaques.* Journal of Virology, 2013. **87**(10): p. 5512-5522.
94. Fang, Y., et al., *Seasonal H1N1 influenza virus infection induces cross-protective pandemic H1N1 virus immunity through a CD8-independent, B cell-dependent mechanism.* Journal of Virology, 2012. **86**(4): p. 2229-2238.
95. Erazo, A., et al., *Unique maturation program of the IgE response in vivo.* Immunity, 2007. **26**(2): p. 191-203.
96. Xiong, H., et al., *Sequential class switching is required for the generation of high affinity IgE antibodies.* Journal of Experimental Medicine, 2012. **209**(2): p. 353-364.
97. O'neill, L.A., D. Golenbock, and A.G. Bowie, *The history of Toll-like receptors--redefining innate immunity.* Nature Reviews Immunology, 2013. **13**(6): p. 453-460.
98. De Nardo, D., *Toll-like receptors: Activation, signalling and transcriptional modulation.* Cytokine, 2015. **74**(2): p. 181-189.
99. Bafica, A., et al., *Cutting edge: TLR9 and TLR2 signaling together account for MyD88-dependent control of parasitemia in Trypanosoma cruzi infection.* Journal of Immunology, 2006. **177**(6): p. 3515-3519.
100. Vasou, A., et al., *Targeting Pattern Recognition Receptors (PRR) for Vaccine Adjuvantation: From Synthetic PRR Agonists to the Potential of Defective Interfering Particles of Viruses.* Viruses, 2017. **9**(7): p. 186-203.
101. Takeda, K., T. Kaisho, and S. Akira, *Toll-like receptors.* Annual review of Immunology, 2003. **21**(1): p. 335-376.
102. Re, F. and J.L. Strominger, *Heterogeneity of TLR-induced responses in dendritic cells: from innate to adaptive immunity.* Immunobiology, 2004. **209**(1): p. 191-198.
103. Pasare, C. and R. Medzhitov, *Control of B-cell responses by Toll-like receptors.* Nature, 2005. **438**(7066): p. 364-368.
104. O'Neill, L.A., C.E. Bryant, and S.L. Doyle, *Therapeutic targeting of Toll-like receptors for infectious and inflammatory diseases and cancer.* Pharmacological Reviews, 2009. **61**(2): p. 177-197.
105. Medzhitov, R., P. Preston-Hurlburt, and C.A. Janeway Jr, *A human homologue of the Drosophila Toll protein signals activation of adaptive immunity.* Nature, 1997. **388**(6640): p. 394-397.
106. Latz, E., et al., *Ligand-induced conformational changes allosterically activate Toll-like receptor 9.* Nature immunology, 2007. **8**(7): p. 772-779.
107. Tanji, H., et al., *Structural reorganization of the Toll-like receptor 8 dimer induced by agonistic ligands.* Science, 2013. **339**(6126): p. 1426-1429.
108. Latz, E., et al., *TLR9 signals after translocating from the ER to CpG DNA in the lysosome.* Nature immunology, 2004. **5**(2): p. 190-198.
109. O'Neill, L.A. and A.G. Bowie, *The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling.* Nature Reviews Immunology, 2007. **7**(5): p. 353-364.
110. Oshiumi, H., et al., *TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-[beta] induction.* Nature immunology, 2003. **4**(2): p. 161-167.
111. Yamamoto, M., et al., *Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway.* Science, 2003. **301**(5633): p. 640-643.
112. Wesche, H., et al., *MyD88: an adapter that recruits IRAK to the IL-1 receptor complex.* Immunity, 1997. **7**(6): p. 837-847.
113. Cheng, S., H. Wang, and H. Zhou, *The Role of TLR4 on B Cell Activation and Anti-GPI Antibody Production in the Antiphospholipid Syndrome.* Journal of Immunology Research, 2016. **2016**: p. 10.
114. Pone, E.J., et al., *B cell Toll-like receptors and immunoglobulin class-switch DNA recombination.* Frontiers in Bioscience (Landmark Ed.), 2012. **17**: p. 2594-2615.
115. Anderson, R. and R. May, *Directly transmitted infections diseases: control by vaccination.* Science, 1982. **215**(4536): p. 1053-1060.

116. Haber, M., J.I.M. Longini, and M.E. Halloran, *Measures of the Effects of Vaccination in a Randomly Mixing Population.* International Journal of Epidemiology, 1991. **20**(1): p. 300-310.
117. Andre, F.E., et al., *Vaccination greatly reduces disease, disability, death and inequity worldwide.* Bulletin of the World Health Organization, 2008. **86**(2): p. 140-146.
118. Schuchat, A., *Human vaccines and their importance to public health.* Procedia in Vaccinology, 2011. **5**: p. 120-126.
119. Plotkin, S.A., W. Orenstein, and P.A. Offit, *Plotkin's Vaccines*. 7th edition, ed. S.A. Plotkin, W. Orenstein, and P.A. Offit. Vol. 2017, Philadelphia: Elsevier. 1720.
120. Ahmed, R. and R.S. Akondy, *Insights into human CD8+ T-cell memory using the yellow fever and smallpox vaccines.* Immunology and Cell Biology, 2011. **89**(3): p. 340-345.
121. Eisen, H.N. and G.W. Siskind, *Variations in affinities of antibodies during the immune response.* Biochemistry, 1964. **3**(7): p. 996-1008.
122. Pulendran, B. and R. Ahmed, *Immunological mechanisms of vaccination.* Nature immunology, 2011. **12**(6): p. 509-517.
123. Plotkin, S.A., *Correlates of protection induced by vaccination.* Clinical and Vaccine Immunology, 2010. **17**(7): p. 1055-1065.
124. Mohsen, M.O., et al., *Major findings and recent advances in virus–like particle (VLP)-based vaccines.* Seminars in Immunology, 2017. **34**: p. 123-132.
125. Skeiky, Y.A. and J.C. Sadoff, *Advances in tuberculosis vaccine strategies.* Nature Reviews Microbiology, 2006. **4**(6): p. 469-477.
126. McMichael, A.J., et al., *The immune response during acute HIV-1 infection: clues for vaccine development.* Nature Reviews Immunology, 2010. **10**(1): p. 11-23.
127. Houghton, M. and S. Abrignani, *Prospects for a vaccine against the hepatitis C virus.* Nature, 2005. **436**(7053): p. 961-966.
128. Langhorne, J., et al., *Immunity to malaria: more questions than answers.* Nature immunology, 2008. **9**(7): p. 725-732.
129. Pawankar, R., *Allergic diseases and asthma: a global public health concern and a call to action.* World Allergy Organization Journal, 2014. **7**(1): p. 12.
130. *World Allergy Organization (WAO) White Book on Allergy: update 2013*, ed. R. Pawankar, et al. Vol. 2013, Milwaukee: World Allergy Organization. 248.
131. Tang, M.L. and R.J. Mullins, *Food allergy: is prevalence increasing?* Internal medicine journal, 2017. **47**(3): p. 256-261.
132. Hoffmann, H.J., et al., *Novel Approaches and Perspectives in Allergen Immunotherapy.* Allergy, 2017. **72**(7): p. 1022-1034.
133. Roberts, G., et al. *EAACI Guidelines on Allergen Immunotherapy: Allergic rhinoconjunctivitis*. [Oct 30 2017]. Allergy. 2017. DOI: 10.1111/all.13317.
134. Jutel, M., et al., *International consensus on allergy immunotherapy.* Journal of Allergy and Clinical Immunology, 2015. **136**(3): p. 556-568.
135. Pajno, G., et al., *Prevention of new sensitizations in asthmatic children monosensitized to house dust mite by specific immunotherapy. A six-year follow-up study.* Clinical & Experimental Allergy, 2001. **31**(9): p. 1392-1397.
136. Dahl, R., et al., *Efficacy and safety of sublingual immunotherapy with grass allergen tablets for seasonal allergic rhinoconjunctivitis.* Journal of Allergy and Clinical Immunology, 2006. **118**(2): p. 434-440.
137. Möller, C., et al., *Pollen immunotherapy reduces the development of asthma in children with seasonal rhinoconjunctivitis (the PAT-study).* Journal of Allergy and Clinical Immunology, 2002. **109**(2): p. 251-256.
138. Schmitt, J., et al., *Allergy immunotherapy for allergic rhinitis effectively prevents asthma: Results from a large retrospective cohort study.* Journal of Allergy and Clinical Immunology, 2015. **136**(6): p. 1511-1516.

139. Niederberger, V., et al., *Vaccination with genetically engineered allergens prevents progression of allergic disease.* Proceedings of the National academy of Sciences, 2004. **101**(suppl 2): p. 14677-14682.
140. Mondoulet, L., et al., *Specific epicutaneous immunotherapy prevents sensitization to new allergens in a murine model.* Journal of Allergy and Clinical Immunology, 2015. **135**(6): p. 1546-1557.
141. Akdis, C.A. and M. Akdis, *Mechanisms of allergen-specific immunotherapy and immune tolerance to allergens.* World Allergy Organization Journal, 2015. **8**(1): p. 17.
142. Casale, T.B. and J.R. Stokes, *Immunotherapy: what lies beyond.* Journal of Allergy and Clinical Immunology, 2014. **133**(3): p. 612-619.
143. Sturm, G.J., et al. *EAACI guidelines on allergen immunotherapy: hymenoptera venom allergy*. [5 Dec 2017]. Allergy. 2017.
144. Senti, G. and T.M. Kündig, *Novel delivery routes for allergy immunotherapy.* Immunology and Allergy Clinics, 2016. **36**(1): p. 25-37.
145. Cox, L.S., et al., *Sublingual immunotherapy: a comprehensive review.* Journal of Allergy and Clinical Immunology, 2006. **117**(5): p. 1021-1035.
146. James, C. and D.I. Bernstein, *Allergen immunotherapy: an updated review of safety.* Current Opinion in Allergy and Clinical Immunology, 2017. **17**(1): p. 55-59.
147. Carlisle, A. and S.M. Jones, *Early Oral Immunotherapy in Peanut-Allergic Preschool Children is Safe and Highly Effective.* Pediatrics, 2017. **140**(Supplement 3): p. S200-S201.
148. Hemler, J.A., *Safety and Efficacy of Low-Dose Oral Immunotherapy for Hen's Egg Allergy in Children.* Pediatrics, 2017. **140**(Supplement 3): p. S202-S203.
149. Brożek, J., et al., *Oral immunotherapy for IgE-mediated cow's milk allergy: a systematic review and meta-analysis.* Clinical & Experimental Allergy, 2012. **42**(3): p. 363-374.
150. Wood, R.A., *Oral immunotherapy for food allergy.* Journal of Investigational Allergology and Clinical Immunology, 2017. **27**(3): p. 151-159.
151. Vazquez-Ortiz, M. and P.J. Turner, *Improving the safety of oral immunotherapy for food allergy.* Pediatric Allergy and Immunology, 2016. **27**(2): p. 117-125.
152. Pajno, G.B., et al. *EAACI Guidelines on Allergen Immunotherapy: IgE-mediated Food Allergy*. [21 Sep 2017]. Allergy. 2017.
153. Jutel, M. and C.A. Akdis, *Novel immunotherapy vaccine development.* Current Opinion in Allergy and Clinical Immunology, 2014. **14**(6): p. 557-563.
154. Senti, G., et al., *Epicutaneous allergen administration as a novel method of allergen-specific immunotherapy.* Journal of Allergy and Clinical Immunology, 2009. **124**(5): p. 997-1002.
155. Martínez-Gómez, J.M., et al., *Intralymphatic injections as a new administration route for allergen-specific immunotherapy.* International Archives of Allergy and Immunology, 2009. **150**(1): p. 59-65.
156. Johansen, P., et al., *Direct intralymphatic injection of peptide vaccines enhances immunogenicity.* European Journal of Immunology, 2005. **35**(2): p. 568-574.
157. Wang, M., et al., *Peanut-induced intestinal allergy is mediated through a mast cell–IgE–FcεRI–IL-13 pathway.* Journal of Allergy and Clinical Immunology, 2010. **126**(2): p. 306-316.
158. Larché, M., C.A. Akdis, and R. Valenta, *Immunological mechanisms of allergen-specific immunotherapy.* Nature Reviews Immunology, 2006. **6**(10): p. 761-771.
159. Jutel, M., et al., *IL-10 and TGF-β cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy.* European Journal of Immunology, 2003. **33**(5): p. 1205-1214.
160. Akdis, C.A., et al., *Epitope-specific T cell tolerance to phospholipase A2 in bee venom immunotherapy and recovery by IL-2 and IL-15 in vitro.* Journal of Clinical Investigation, 1996. **98**(7): p. 1676-1683.
161. Woo, H.Y., et al., *Mechanism for acute oral desensitization to antibiotics.* Allergy, 2006. **61**(8): p. 954-958.

162. Plewako, H., et al., *Basophil interleukin 4 and interleukin 13 production is suppressed during the early phase of rush immunotherapy.* International Archives of Allergy and Immunology, 2006. **141**(4): p. 346-353.
163. Suárez-Fueyo, A., et al., *Grass tablet sublingual immunotherapy downregulates the T H 2 cytokine response followed by regulatory T-cell generation.* Journal of Allergy and Clinical Immunology, 2014. **133**(1): p. 130-138.
164. Nouri-Aria, K.T., et al., *Grass pollen immunotherapy induces mucosal and peripheral IL-10 responses and blocking IgG activity.* Journal of Immunology, 2004. **172**(5): p. 3252-3259.
165. Ree, R., et al., *Measurement of IgE antibodies against purified grass pollen allergens (Lol p 1, 2, 3 and 5) during immunotherapy.* Clinical & Experimental Allergy, 1997. **27**(1): p. 68-74.
166. Shamji, M., et al. *Biomarkers for monitoring clinical efficacy of allergen immunotherapy for allergic rhinoconjunctivitis and allergic asthma: an EAACI Position Paper*. [Journal on the internet]. [Apr 6 2017]. Allergy. 2017. DOI: 10.1111/all.13138.
167. Shamji, M.H., et al., *Cell-free detection of allergen-IgE cross-linking with immobilized phase CD23: inhibition by blocking antibody responses after immunotherapy.* Journal of Allergy and Clinical Immunology, 2013. **132**(4): p. 1003-1005.
168. Shamji, M., et al., *Functional rather than immunoreactive levels of IgG4 correlate closely with clinical response to grass pollen immunotherapy.* Allergy, 2012. **67**(2): p. 217-226.
169. Shamji, M.H., et al., *The IgE-facilitated allergen binding (FAB) assay: Validation of a novel flow-cytometric based method for the detection of inhibitory antibody responses.* Journal of Immunological Methods, 2006. **317**(1): p. 71-79.
170. van Neerven, R.J.J., et al., *Blocking Antibodies Induced by Specific Allergy Vaccination Prevent the Activation of CD4+ T Cells by Inhibiting Serum-IgE-Facilitated Allergen Presentation.* Journal of Immunology, 1999. **163**(5): p. 2944-2952.
171. Patel, D., et al., *Fel d 1–derived peptide antigen desensitization shows a persistent treatment effect 1 year after the start of dosing: A randomized, placebo-controlled study.* Journal of Allergy and Clinical Immunology, 2013. **131**(1): p. 103-109.
172. Larche, M., *Peptide immunotherapy for allergic diseases.* Allergy, 2007. **62**(3): p. 325-331.
173. Spertini, F., et al., *Safety and immunogenicity of immunotherapy with Bet v 1–derived contiguous overlapping peptides.* Journal of Allergy and Clinical Immunology, 2014. **134**(1): p. 239-240.
174. Valenta, R., et al., *Recombinant allergens: what does the future hold?* Journal of Allergy and Clinical Immunology, 2011. **127**(4): p. 860-864.
175. Valenta, R., et al., *Vaccine development for allergen-specific immunotherapy based on recombinant allergens and synthetic allergen peptides: Lessons from the past and novel mechanisms of action for the future.* Journal of Allergy and Clinical Immunology, 2016. **137**(2): p. 351-357.
176. Mösges, R., et al. *A randomized, double-blind, placebo-controlled, dose-finding trial with Lolium perenne peptide immunotherapy*. [Journal on the internet]. [Dec 22 2017]. Allergy. 2017. DOI: 10.1111/all.13358.
177. Couroux, P., et al., *Fel d 1-derived synthetic peptide immuno-regulatory epitopes show a long-term treatment effect in cat allergic subjects.* Clinical & Experimental Allergy, 2015. **45**(5): p. 974-981.
178. Pellaton, C., et al., *Novel birch pollen specific immunotherapy formulation based on contiguous overlapping peptides.* Clinical and translational allergy, 2013. **3**(1): p. 17.
179. Focke-Tejkl, M., et al., *Development and characterization of a recombinant, hypoallergenic, peptide-based vaccine for grass pollen allergy.* Journal of Allergy and Clinical Immunology, 2015. **135**(5): p. 1207-1217.
180. Sallusto, F., et al., *From vaccines to memory and back.* Immunity, 2010. **33**(4): p. 451-463.
181. Oiseth, S.J. and M.S. Aziz, *Cancer immunotherapy: a brief review of the history, possibilities, and challenges ahead.* Journal of Cancer Metastasis and Treatment, 2017. **3**: p. 250-261.
182. Neefjes, J., et al., *Towards a systems understanding of MHC class I and MHC class II antigen presentation.* Nature Reviews Immunology, 2011. **11**(12): p. 823-836.

183. Zhang, S., H. Zhang, and J. Zhao, *The role of CD4 T cell help for CD8 CTL activation.* Biochemical and biophysical research communications, 2009. **384**(4): p. 405-408.
184. Copland, M.J., et al., *Lipid based particulate formulations for the delivery of antigen.* Immunology and Cell Biology, 2005. **83**(2): p. 97-105.
185. Brito, L.A. and D.T. O'Hagan, *Designing and building the next generation of improved vaccine adjuvants.* Journal of Controlled Release, 2014. **190**: p. 563-579.
186. Shah, R.R., K.J. Hassett, and L.A. Brito, *Overview of Vaccine Adjuvants: Introduction, History, and Current Status*, in *Vaccine adjuvants. Methods in Molecular Biology*, C.B. Fox, Editor. Vol. 1494. 2017, Humana Press: New York, NY. p. 1-13.
187. Dowling, D.J. and O. Levy, *Pediatric Vaccine Adjuvants: Components of the Modern Vaccinologist's Toolbox.* The Pediatric Infectious Disease Journal, 2015. **34**(12): p. 1395-1398.
188. Brito, L.A., P. Malyala, and D.T. O'Hagan, *Vaccine adjuvant formulations: a pharmaceutical perspective.* Seminars in Immunology, 2013. **25**(2): p. 130-145.
189. Rambe, D.S., et al., *Safety and mechanism of action of licensed vaccine adjuvants.* International Current Pharmaceutical Journal, 2015. **4**(8): p. 420-431.
190. Cimica, V. and J.M. Galarza, *Adjuvant formulations for virus-like particle (VLP) based vaccines.* Clinical Immunology, 2017. **183**: p. 99-108.
191. Noe, S.M., et al., *Mechanism of immunopotentiation by aluminum-containing adjuvants elucidated by the relationship between antigen retention at the inoculation site and the immune response.* Vaccine, 2010. **28**(20): p. 3588-3594.
192. Marichal, T., et al., *DNA released from dying host cells mediates aluminum adjuvant activity.* Nature medicine, 2011. **17**(8): p. 996-1002.
193. Tritto, E., F. Mosca, and E. De Gregorio, *Mechanism of action of licensed vaccine adjuvants.* Vaccine, 2009. **27**(25): p. 3331-3334.
194. Bachmann, M.F. and G.T. Jennings, *Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns.* Nature Reviews Immunology, 2010. **10**(11): p. 787-796.
195. Flach, T.L., et al., *Alum interaction with dendritic cell membrane lipids is essential for its adjuvanticity.* Nature medicine, 2011. **17**(4): p. 479-487.
196. Gavin, A.L., et al., *Adjuvant-enhanced antibody responses in the absence of toll-like receptor signaling.* Science, 2006. **314**(5807): p. 1936-1938.
197. Jordan, M.B., et al., *Promotion of B cell immune responses via an alum-induced myeloid cell population.* Science, 2004. **304**(5678): p. 1808-1810.
198. Didierlaurent, A.M., et al., *AS04, an aluminum salt-and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity.* Journal of Immunology, 2009. **183**(10): p. 6186-6197.
199. Mothes, N., et al., *Allergen-specific immunotherapy with a monophosphoryl lipid A-adjuvanted vaccine: reduced seasonally boosted immunoglobulin E production and inhibition of basophil histamine release by therapy-induced blocking antibodies.* Clinical & Experimental Allergy, 2003. **33**(9): p. 1198-1208.
200. Parnham, M.J. and H. Wetzig, *Toxicity screening of liposomes.* Chemistry and physics of lipids, 1993. **64**(1-3): p. 263-274.
201. Felnerova, D., et al., *Liposomes and virosomes as delivery systems for antigens, nucleic acids and drugs.* Current Opinion in Biotechnology, 2004. **15**(6): p. 518-529.
202. Cheng, W., *The density code for the development of a vaccine?* Journal of Pharmaceutical Sciences, 2016. **105**(11): p. 3223-3232.
203. Kasturi, S.P., et al., *Programming the magnitude and persistence of antibody responses with innate immunity.* Nature, 2011. **470**(7335): p. 543-547.
204. De Serrano, L.O. and D.J. Burkhart, *Liposomal vaccine formulations as prophylactic agents: design considerations for modern vaccines.* Journal of Nanobiotechnology, 2017. **15**(1): p. 83.
205. Miao, L., et al., *Enhanced Immune Response to Rabies Viruses by the Use of a Liposome Adjuvant in Vaccines.* Viral Immunology, 2017. **30**(10): p. 727-733.

206. Zamani, P., et al. *Nanoliposomes as the adjuvant delivery systems in cancer immunotherapy*. [Journal on the internet]. [Dec 7 2017]. Journal of Cellular Physiology. 2017. DOI: 10.1002/jcp.26361.
207. van Dissel, J.T., et al., *A novel liposomal adjuvant system, CAF01, promotes long-lived Mycobacterium tuberculosis-specific T-cell responses in human.* Vaccine, 2014. **32**(52): p. 7098-7107.
208. Melero, I., et al., *Therapeutic vaccines for cancer: an overview of clinical trials.* Nature Reviews Clinical Oncology, 2014. **11**(9): p. 509-524.
209. Agnandji, S.T., et al., *Clinical development of RTS, S/AS malaria vaccine: a systematic review of clinical Phase I–III trials.* Future Microbiology, 2015. **10**(10): p. 1553-1578.
210. van Kasteren, S.I., et al., *Chemical biology of antigen presentation by MHC molecules.* Current Opinion in Immunology, 2014. **26**: p. 21-31.
211. Taylor, J.J., K.A. Pape, and M.K. Jenkins, *A germinal center–independent pathway generates unswitched memory B cells early in the primary response.* Journal of Experimental Medicine, 2012. **209**(3): p. 597-606.
212. Kaji, T., et al., *Distinct cellular pathways select germline-encoded and somatically mutated antibodies into immunological memory.* Journal of Experimental Medicine, 2012. **209**(11): p. 2079-2097.
213. Gitlin, A.D., et al., *Independent roles of switching and hypermutation in the development and persistence of B lymphocyte memory.* Immunity, 2016. **44**(4): p. 769-781.
214. Kräutler, N.J., et al., *Differentiation of germinal center B cells into plasma cells is initiated by high-affinity antigen and completed by Tfh cells.* Journal of Experimental Medicine, 2017. **214**(5): p. 1259-1267.
215. Belichenko, P.V., et al., *An anti-β-amyloid vaccine for treating cognitive deficits in a mouse model of down syndrome.* PloS one, 2016. **11**(3): p. e0152471.
216. Kahlert, H., et al., *Epitope analysis of the allergen ovalbumin (Gal d II) with monoclonal antibodies and patients' IgE.* Molecular Immunology, 1992. **29**(10): p. 1191-1201.
217. Mine, Y. and M. Yang, *Epitope characterization of ovalbumin in BALB/c mice using different entry routes.* Biochimica et Biophysica Acta, 2007. **1774**(2): p. 200-212.
218. Yang, M. and Y. Mine, *Novel T-cell epitopes of ovalbumin in BALB/c mouse: potential for peptide-immunotherapy.* Biochemical and biophysical research communications, 2009. **378**(2): p. 203-208.
219. Mackenzie, K.J., et al., *Combination peptide immunotherapy based on T-cell epitope mapping reduces allergen-specific IgE and eosinophilia in allergic airway inflammation.* Immunology, 2013. **138**(3): p. 258-268.
220. Mine, Y. and P. Rupa, *Fine mapping and structural analysis of immunodominant IgE allergenic epitopes in chicken egg ovalbumin.* Protein Engineering, 2003. **16**(10): p. 747-752.
221. Yang, M., C. Yang, and Y. Mine, *Multiple T cell epitope peptides suppress allergic responses in an egg allergy mouse model by the elicitation of forkhead box transcription factor 3-and transforming growth factor-β-associated mechanisms.* Clinical & Experimental Allergy, 2010. **40**(4): p. 668-678.
222. Yang, M., et al., *Immunomodulatory effects of egg white enzymatic hydrolysates containing immunodominant epitopes in a BALB/c mouse model of egg allergy.* Journal of Agricultural and Food Chemistry, 2009. **57**(6): p. 2241-2248.
223. López-Expósito, I., et al., *Changes in the ovalbumin proteolysis profile by high pressure and its effect on IgG and IgE binding.* Journal of Agricultural and Food Chemistry, 2008. **56**(24): p. 11809-11816.
224. Muhs, A., et al., *Liposomal vaccines with conformation-specific amyloid peptide antigens define immune response and efficacy in APP transgenic mice.* Proceedings of the National Academy of Sciences of the United States of America, 2007. **104**(23): p. 9810-9815.
225. Idicula-Thomas, S. and P.V. Balaji, *Understanding the relationship between the primary structure of proteins and its propensity to be soluble on overexpression in Escherichia coli.* Protein Science, 2005. **14**(3): p. 582-592.

226. Johansen, P., et al., *Heat denaturation, a simple method to improve the immunotherapeutic potential of allergens.* European Journal of Immunology, 2005. **35**(12): p. 3591-3598.
227. Devey, M., et al., *Determination of the functional affinity of IgG1 and IgG4 antibodies to tetanus toxoid by isotype-specific solid-phase assays.* Journal of Immunological Methods, 1988. **106**(1): p. 119-125.
228. Pihlgren, M., et al., *TLR4- and TRIF-dependent stimulation of B lymphocytes by peptide liposomes enables T cell–independent isotype switch in mice.* Blood, 2013. **121**(1): p. 85-94.
229. Men, Y., et al., *A single administration of tetanus toxoid in biodegradable microspheres elicits T cell and antibody responses similar or superior to those obtained with aluminum hydroxide.* Vaccine, 1995. **13**(7): p. 683-689.
230. Zabel, F., T.M. Kündig, and M.F. Bachmann, *Virus-induced humoral immunity: on how B cell responses are initiated.* Current Opinion in Virology, 2013. **3**(3): p. 357-362.
231. Finkelman, F.D., *Anaphylaxis: lessons from mouse models.* Journal of Allergy and Clinical Immunology, 2007. **120**(3): p. 506-515.
232. Zehn, D., S.Y. Lee, and M.J. Bevan, *Complete but curtailed T cell response to very low affinity antigen.* Nature, 2009. **458**(7235): p. 211-214.
233. Bioley, G., et al., *The effect of vaccines based on ovalbumin coupled to gas-filled microbubbles for reducing infection by ovalbumin-expressing Listeria monocytogenes.* Biomaterials, 2013. **34**(21): p. 5423-5430.
234. Bachmann, M.F., H. Hengartner, and R.M. Zinkernagel, *T helper cell-independent neutralizing B cell response against vesicular stomatitis virus: Role of antigen patterns in B cell induction?* European Journal of Immunology, 1995. **25**(12): p. 3445-3451.
235. Sulzer, B. and A.S. Perelson, *Immunons revisited: binding of multivalent antigens to B cells.* Molecular Immunology, 1997. **34**(1): p. 63-74.
236. Bachmann, M.F., et al., *The influence of antigen organization on B cell responsiveness.* Science, 1993. **262**(5138): p. 1448-1451.
237. Jegerlehner, A., et al., *Regulation of IgG antibody responses by epitope density and CD21-mediated costimulation.* European Journal of Immunology, 2002. **32**(11): p. 3305-3314.
238. Szomolanyi-Tsuda, E., et al., *T-cell-independent immunoglobulin G responses in vivo are elicited by live-virus infection but not by immunization with viral proteins or virus-like particles.* Journal of Virology, 1998. **72**(8): p. 6665-6670.
239. Raval, F., et al., *Long-lasting T cell-independent IgG responses require MyD88-mediated pathways and are maintained by high levels of virus persistence.* MBio, 2013. **4**(6): p. e00812-13.
240. Minguet, S., et al., *Enhanced B-cell activation mediated by TLR4 and BCR crosstalk.* European Journal of Immunology, 2008. **38**(9): p. 2475-2487.
241. Schweighoffer, E., et al., *TLR4 signals in B lymphocytes are transduced via the B cell antigen receptor and SYK.* Journal of Experimental Medicine, 2017. **214**(5): p. 1269-1280.
242. Persing, D.H., et al., *Taking toll: lipid A mimetics as adjuvants and immunomodulators.* Trends in Microbiology, 2002. **10**(10): p. s32-s37.
243. Jiang, Z., et al., *CD14 is required for MyD88-independent LPS signaling.* Nature immunology, 2005. **6**(6): p. 565-570.
244. Zanoni, I., et al., *CD14 controls the LPS-induced endocytosis of Toll-like receptor 4.* Cell, 2011. **147**(4): p. 868-880.
245. Yanagibashi, T., et al., *Differential requirements of MyD88 and TRIF pathways in TLR4-mediated immune responses in murine B cells.* Immunology letters, 2015. **163**(1): p. 22-31.
246. Barrio, L., J.S. de Guinoa, and Y.R. Carrasco, *TLR4 signaling shapes B cell dynamics via MyD88-dependent pathways and Rac GTPases.* Journal of Immunology, 2013. **191**(7): p. 3867-3875.
247. Janssen, E., et al., *TRIF signaling is essential for TLR4-driven IgE class switching.* Journal of Immunology, 2014. **192**(6): p. 2651-2658.
248. Mata-Haro, V., et al., *The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4.* Science, 2007. **316**(5831): p. 1628-1632.

249. Heise, N., et al., *Germinal center B cell maintenance and differentiation are controlled by distinct NF-κB transcription factor subunits.* Journal of Experimental Medicine, 2014. **211**(10): p. 2103-2118.
250. Di Niro, R., et al., *Salmonella infection drives promiscuous B cell activation followed by extrafollicular affinity maturation.* Immunity, 2015. **43**(1): p. 120-131.
251. Higdon, L.E. and M.P. Cancro, *Caught Off Center: Rethinking the Requirements for Antibody Affinity Maturation.* Immunity, 2015. **43**(1): p. 5-6.
252. Han, J.-H., et al., *Class switch recombination and somatic hypermutation in early mouse B cells are mediated by B cell and Toll-like receptors.* Immunity, 2007. **27**(1): p. 64-75.
253. Chaplin, J.W., C.P. Chappell, and E.A. Clark, *Targeting antigens to CD180 rapidly induces antigen-specific IgG, affinity maturation, and immunological memory.* Journal of Experimental Medicine, 2013. **210**(10): p. 2135-2146.
254. Rubin, L.G., et al., *2013 IDSA clinical practice guideline for vaccination of the immunocompromised host.* Clinical Infectious Diseases, 2013. **58**(3): p. e44-e100.
255. Shearer, W.T., et al., *Recommendations for live viral and bacterial vaccines in immunodeficient patients and their close contacts.* Journal of Allergy and Clinical Immunology, 2014. **133**(4): p. 961-966.
256. Lemere, C.A. and E. Masliah, *Can Alzheimer disease be prevented by amyloid-β immunotherapy?* Nature Reviews Neurology, 2010. **6**(2): p. 108-119.
257. Dorfmeier, C.L., et al., *Protective Vaccine-Induced CD4+ T Cell-Independent B Cell Responses against Rabies Infection.* Journal of Virology, 2012. **86**(21): p. 11533-11540.
258. Rolland, J. and R. O'Hehir, *Immunotherapy of allergy: anergy, deletion, and immune deviation.* Current Opinion in Immunology, 1998. **10**(6): p. 640-645.
259. Flicker, S., et al., *Passive immunization with allergen-specific IgG antibodies for treatment and prevention of allergy.* Immunobiology, 2013. **218**(6): p. 884-891.
260. Waeckerle-Men, Y., et al., *Multivalent paediatric allergy vaccines protect against allergic anaphylaxis in mice.* Clinical & Experimental Allergy, 2014. **44**(3): p. 429-437.
261. Burks, A.W., et al., *Update on allergy immunotherapy: American academy of allergy, asthma & immunology/European academy of allergy and clinical immunology/PRACTALL consensus report.* Journal of Allergy and Clinical Immunology, 2013. **131**(5): p. 1288-1296. e3.
262. James, L.K., et al., *Long-term tolerance after allergen immunotherapy is accompanied by selective persistence of blocking antibodies.* Journal of Allergy and Clinical Immunology, 2011. **127**(2): p. 509-516.
263. Jilek, S., et al., *Antigen-independent suppression of the allergic immune response to bee venom phospholipase A2 by DNA vaccination in CBA/J mice.* Journal of Immunology, 2001. **166**(5): p. 3612-3621.
264. Maazi, H., et al., *Contribution of regulatory T cells to alleviation of experimental allergic asthma after specific immunotherapy.* Clinical & Experimental Allergy, 2012. **42**(10): p. 1519-1528.
265. Umetsu, D.T., et al., *Regulatory T cells control the development of allergic disease and asthma.* Journal of Allergy and Clinical Immunology, 2003. **112**(3): p. 480-487.
266. Schmitz, N., et al., *Displaying Fel d1 on virus-like particles prevents reactogenicity despite greatly enhanced immunogenicity: a novel therapy for cat allergy.* Journal of Experimental Medicine, 2009. **206**(9): p. 1941-1955.
267. Hjálmsdóttir, Á., et al., *Dosing intervals in intralymphatic immunotherapy.* Clinical & Experimental Allergy, 2016. **46**(3): p. 504-507.
268. Castle, P.E. and M. Maza, *Prophylactic HPV vaccination: past, present, and future.* Epidemiology and Infection, 2015. **144**(3): p. 449-468.
269. Walker, R.D., et al., *Recommendations for preventive pediatric health care.* Pediatrics, 2007. **120**(6): p. 1376.
270. Van Buren, R.C. and W. Schaffner, *Hepatitis B virus: a comprehensive strategy for eliminating transmission in the United States through universal childhood vaccination: recommendations of the*

Immunization Practices Advisory Committee (ACIP). MMWR Recommended Reports, 1991. **40**(RR-13): p. 1-25.
271. Steiner, R.P., et al., *Minimum time interval adjustment for 4-3-1 immunization rates among two-year-old children.* American journal of preventive medicine, 1999. **16**(3): p. 189-194.
272. Goidl, E.A., et al., *The effect of antigen dose and time after immunization on the amount and affinity of anti-hapten antibody.* Journal of Immunology, 1968. **100**: p. 371-375.
273. Senti, G., et al., *Intralymphatic immunotherapy for cat allergy induces tolerance after only 3 injections.* Journal of Allergy and Clinical Immunology, 2012. **129**(5): p. 1290-1296.
274. Senti, G., et al., *Intralymphatic allergen administration renders specific immunotherapy faster and safer: a randomized controlled trial.* Proceedings of the National Academy of Sciences, 2008. **105**(46): p. 17908-17912.
275. Hylander, T., et al., *Intralymphatic allergen-specific immunotherapy: an effective and safe alternative treatment route for pollen-induced allergic rhinitis.* Journal of Allergy and Clinical Immunology, 2013. **131**(2): p. 412-420.
276. Witten, M., et al., *Is intralymphatic immunotherapy ready for clinical use in patients with grass pollen allergy?* Journal of Allergy and Clinical Immunology, 2013. **132**(5): p. 1248-1252.
277. Kedl, R.M., J.W. Kappler, and P. Marrack, *Epitope dominance, competition and T cell affinity maturation.* Current Opinion in Immunology, 2003. **15**(1): p. 120-127.
278. Martineau, P., *Affinity measurements by competition ELISA*, in *Antibody engineering*, R. Kontermann and S. Dübel, Editors. Vol. 2010, Springer-Verlag: Berlin Heidelberg. p. 657-665.
279. Ni, J., et al., *Toward the next generation of NK cell-based adoptive cancer immunotherapy.* Oncoimmunology, 2013. **2**(4): p. e23811.
280. Phan, G.Q. and S.A. Rosenberg, *Adoptive cell transfer for patients with metastatic melanoma: the potential and promise of cancer immunotherapy.* Cancer Control, 2013. **20**(4): p. 289-97.
281. Hinrichs, C.S. and S.A. Rosenberg, *Exploiting the curative potential of adoptive T-cell therapy for cancer.* Immunol Rev, 2014. **257**(1): p. 56-71.
282. Kalos, M. and C.H. June, *Adoptive T cell transfer for cancer immunotherapy in the era of synthetic biology.* Immunity, 2013. **39**(1): p. 49-60.
283. Goldman, B. and L. DeFrancesco, *The cancer vaccine roller coaster.* Nat Biotechnol, 2009. **27**(2): p. 129-39.
284. Ridolfi, L., et al., *Dendritic cell-based vaccine in advanced melanoma: update of clinical outcome.* Melanoma Res, 2011. **21**(6): p. 524-9.
285. Carreno, B.M., et al., *IL-12p70-producing patient DC vaccine elicits Tc1-polarized immunity.* J Clin Invest, 2013. **123**(8): p. 3383-94.
286. Yamada, A., et al., *Next-generation peptide vaccines for advanced cancer.* Cancer Sci, 2013. **104**(1): p. 15-21.
287. Mathew, E., et al., *Cytosolic delivery of antisense oligonucleotides by listeriolysin O-containing liposomes.* Gene Ther, 2003. **10**(13): p. 1105-15.
288. Banchereau, J. and A.K. Palucka, *Dendritic cells as therapeutic vaccines against cancer.* Nat Rev Immunol, 2005. **5**(4): p. 296-306.
289. Gilboa, E., *DC-based cancer vaccines.* J Clin Invest, 2007. **117**(5): p. 1195-203.
290. O'Neill, D.W., S. Adams, and N. Bhardwaj, *Manipulating dendritic cell biology for the active immunotherapy of cancer.* Blood, 2004. **104**(8): p. 2235-46.
291. Reddy, S.T., M.A. Swartz, and J.A. Hubbell, *Targeting dendritic cells with biomaterials: developing the next generation of vaccines.* Trends Immunol, 2006. **27**(12): p. 573-9.
292. Tacken, P.J., et al., *Dendritic-cell immunotherapy: from ex vivo loading to in vivo targeting.* Nat Rev Immunol, 2007. **7**(10): p. 790-802.
293. Kaech, S.M. and R. Ahmed, *Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naive cells.* Nat Immunol, 2001. **2**(5): p. 415-22.
294. Lehner, P.J. and P. Cresswell, *Recent developments in MHC-class-I-mediated antigen presentation.* Curr Opin Immunol, 2004. **16**(1): p. 82-9.

295. Princiotta, M.F., et al., *Quantitating protein synthesis, degradation, and endogenous antigen processing.* Immunity, 2003. **18**(3): p. 343-54.
296. Yewdell, J.W., E. Reits, and J. Neefjes, *Making sense of mass destruction: quantitating MHC class I antigen presentation.* Nat Rev Immunol, 2003. **3**(12): p. 952-61.
297. Jarver, P. and U. Langel, *Cell-penetrating peptides--a brief introduction.* Biochim Biophys Acta, 2006. **1758**(3): p. 260-3.
298. Martinez Gomez, J.M., et al., *Surface coating of PLGA microparticles with protamine enhances their immunological performance through facilitated phagocytosis.* J Control Release, 2008. **130**(2): p. 161-7.
299. Park, Y.J., et al., *Nontoxic membrane translocation peptide from protamine, low molecular weight protamine (LMWP), for enhanced intracellular protein delivery: in vitro and in vivo study.* FASEB J, 2005. **19**(11): p. 1555-7.
300. Reynolds, F., R. Weissleder, and L. Josephson, *Protamine as an efficient membrane-translocating peptide.* Bioconjug Chem, 2005. **16**(5): p. 1240-5.
301. Karlsen, K., et al., *A stable nanoparticulate DDA/MMG formulation acts synergistically with CpG ODN 1826 to enhance the CD4(+) T-cell response.* Nanomedicine (Lond), 2014. **9**(17): p. 2625-38.
302. Slutter, B., et al., *Adjuvant effect of cationic liposomes and CpG depends on administration route.* J Control Release, 2011. **154**(2): p. 123-30.
303. Karanth, H. and R.S. Murthy, *pH-sensitive liposomes--principle and application in cancer therapy.* J Pharm Pharmacol, 2007. **59**(4): p. 469-83.
304. Korsholm, K.S., et al., *Induction of CD8+ T-cell responses against subunit antigens by the novel cationic liposomal CAF09 adjuvant.* Vaccine, 2014. **32**(31): p. 3927-35.
305. Nordly, P., et al., *Immunity by formulation design: induction of high CD8+ T-cell responses by poly(I:C) incorporated into the CAF01 adjuvant via a double emulsion method.* J Control Release, 2011. **150**(3): p. 307-17.
306. Johansen, P., et al., *Relief from Zmp1-mediated arrest of phagosome maturation is associated with facilitated presentation and enhanced immunogenicity of mycobacterial antigens.* Clin Vaccine Immunol, 2011. **18**(6): p. 907-13.
307. Grode, L., et al., *Increased vaccine efficacy against tuberculosis of recombinant Mycobacterium bovis bacille Calmette-Guerin mutants that secrete listeriolysin.* J Clin Invest, 2005. **115**(9): p. 2472-9.
308. Sun, R., et al., *Novel recombinant BCG expressing perfringolysin O and the over-expression of key immunodominant antigens; pre-clinical characterization, safety and protection against challenge with Mycobacterium tuberculosis.* Vaccine, 2009. **27**(33): p. 4412-23.
309. Chen, J.L., et al., *NY-ESO-1 specific antibody and cellular responses in melanoma patients primed with NY-ESO-1 protein in ISCOMATRIX and boosted with recombinant NY-ESO-1 fowlpox virus.* Int J Cancer, 2015. **136**(6): p. E590-601.
310. Morelli, A.B., et al., *ISCOMATRIX: a novel adjuvant for use in prophylactic and therapeutic vaccines against infectious diseases.* J Med Microbiol, 2012. **61**(Pt 7): p. 935-43.
311. Wilson, N.S., et al., *ISCOMATRIX vaccines mediate CD8+ T-cell cross-priming by a MyD88-dependent signaling pathway.* Immunol Cell Biol, 2012. **90**(5): p. 540-52.
312. Shiver, J.W. and E.A. Emini, *Recent advances in the development of HIV-1 vaccines using replication-incompetent adenovirus vectors.* Annu Rev Med, 2004. **55**: p. 355-72.
313. Tatsis, N. and H.C. Ertl, *Adenoviruses as vaccine vectors.* Mol Ther, 2004. **10**(4): p. 616-29.
314. Spreafico, R., P. Ricciardi-Castagnoli, and A. Mortellaro, *The controversial relationship between NLRP3, alum, danger signals and the next-generation adjuvants.* Eur J Immunol, 2010. **40**(3): p. 638-42.
315. O'Hagan, D.T. and C.B. Fox, *New generation adjuvants--from empiricism to rational design.* Vaccine, 2015. **33 Suppl 2**: p. B14-20.
316. Obeid, J., Y. Hu, and C.L. Slingluff, Jr., *Vaccines, Adjuvants, and Dendritic Cell Activators-Current Status and Future Challenges.* Semin Oncol, 2015. **42**(4): p. 549-61.

317. Powell, B.S., A.K. Andrianov, and P.C. Fusco, *Polyionic vaccine adjuvants: another look at aluminum salts and polyelectrolytes.* Clin Exp Vaccine Res, 2015. **4**(1): p. 23-45.
318. Håkerud, M., et al., *Intradermal photosensitisation facilitates stimulation of MHC class-I restricted CD8 T-cell responses of co-administered antigen.* J Control Release, 2014. **174**: p. 143-50.
319. Waeckerle-Men, Y., et al., *Photochemical targeting of antigens to the cytosol for stimulation of MHC class-I-restricted T-cell responses.* Eur J Pharm Biopharm, 2013. **85**(1): p. 34-41.
320. Håkerud, M., et al., *Photosensitisation facilitates cross-priming of adjuvant-free protein vaccines and stimulation of tumour-suppressing CD8 T cells.* Journal of Controlled Release, 2015. **198**: p. 10-17.
321. Oppenheim, J.J., *IL-2: more than a T cell growth factor.* J Immunol, 2007. **179**(3): p. 1413-4.
322. Hofer, T., O. Krichevsky, and G. Altan-Bonnet, *Competition for IL-2 between Regulatory and Effector T Cells to Chisel Immune Responses.* Front Immunol, 2012. **3**: p. 268.
323. Faria, A.M.C. and H.L. Weiner, *Oral Tolerance: Mechanisms and Therapeutic Applications.* Advances in Immunology, 1999. **73**: p. 153-264.